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Chemistry and biochemistry of benzoxazinones in maize

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Chemistry and biochemistry of benzoxazinones in maize

by

Francis Hsiang-Chian Tsao

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TABLE OF CONTENTS

INTRODUCTION

One of the benzoxazinones, 2,4-dihydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one (DIMBOA), a naturally occurring cyclic hydroxamic acid in maize, has been studied extensively due to its agronomic importance. This cyclic hydroxamic acid has been reported to confer resistance to the European corn borer, leaf blight, stalk rot, and stem rust, and tolerance of certain plants to 2-chloro-s-triazine herbicides.

The content of DIMBOA in maize plays a very important role in the resistance to the corn borer. The variety which has a high concentration of DIMBOA has high resistance and the one with low concentration is susceptible. The concentration of DIMBOA drops while the plant grows. This correlation has brought impetus to investigations of the genetic and biochemical mechanism controlling the DIMBOA concentration.

The biologically important functional group, hydroxamate, has been studied by many researchers not only for its biological significance, but also because of the chemical characteristics. The study of the biosynthesis of benzoxazinones in plants is only at an early stage and is not well known. The chemical synthesis of the cyclic hydroxamic acid, 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) in good yield has not been achieved previously although there are several potential uses for this material if available in sufficient quantities.

The study in this dissertation covers:

(1) The investigation of the biosynthesis of DIMBOA by feeding experiments and in cell-free extracts.

(2) Measurement of the turn-over rate of DIMBOA and correlation with

the rates of some reactions which might be involved in the degradation of the benzoxazinones.

(3) Chemical aspects of the detoxification of simazine (2-chloro-4,6-bis-(ethylamino)-s-triazine) catalyzed by DIMBOA.

(4) Study of the degradation of DIMBOA in acid as a model for the enzymatic interconversion of the benzoxazinones.

(5) An efficient, economical chemical procedure to synthesize the cyclic hydroxamic acids.

REVIEW OF LITERATURE

The most effective and ideal method for reducing economic cost of insects that attack plants is to grow insect-resistant varieties. It is well known that plant tissues may contain substances which deter insect attack. The development of inbred lines of maize resistant or tolerant to the European corn borer has been in progress for about 25 years. In 1953, Beck (1) observed that two or more substances which occur in corn plant tissues have a deleterious effect on the growth and survival of borer larvae. One of the substances was isolated in pure form and was called Resistance Factor A (2). This Factor A was later identified as 6-methoxy-benzoxazolinone (6MBOA) (3, 4). Virtanen and Heitala (5) also identified an antifusarium factor formed in rye seedlings as benzoxazolinone (BOA).

It was later indicated by Wahlroos and Virtanen (6) that both 6MBOA isolated from wheat and maize plants and BOA isolated from rye plants were not constituents of plant tissues *in vivo*. In the intact plants, the precursors exist predominantly as monoglucosides which are hydrolyzed to the aglucones, 2,4-dihydroxy compounds (cyclic hydroxamic acids) following mechanical injury to the cells (7, 8). The aglucone also occurs at a low concentration in intact maize plants (9). The cyclic hydroxamic acids are unstable and undergo quantitative decomposition to the corresponding benzoxazolinones and formic acid (6, 10). The reaction in water follows first-order kinetics and is dependent on the concentration of the anion of the compound (11). A similar reaction was also found to occur by heating of 4-hydroxy-1,4-benzoxazine-2,3-dione, carbon dioxide being split off

(12). However, the lactam forms of the 1,4-benzoxazin-3-one compounds can not be decomposed to form the corresponding benzoxazolinones. This is due to the presence of a N-hydroxyl group and an easily ruptured O(1) - C(2) bond being a prerequisite for this reaction. The lactam forms were first obtained by reducing the cyclic hydroxamic acids with zinc dust in boiling glacial acetic acid (13). It was found recently that the lactams are also natural products, occurring as glucosides in maize (14, 15). It was also observed recently that the cyclic hydroxamic acid and lactam forms of the 1,4-benzoxazin-3-one compounds are readily interconverted in vivo (16). DIMBOA-glucoside (2,4-dihydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one-glucoside) and HMBOA-glucoside (2-hydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one-glucoside) were suggested to be interconverted metabolically (17). Structures of cyclic hydroxamic acids and related compounds found in young maize plants are shown in Figure 1.

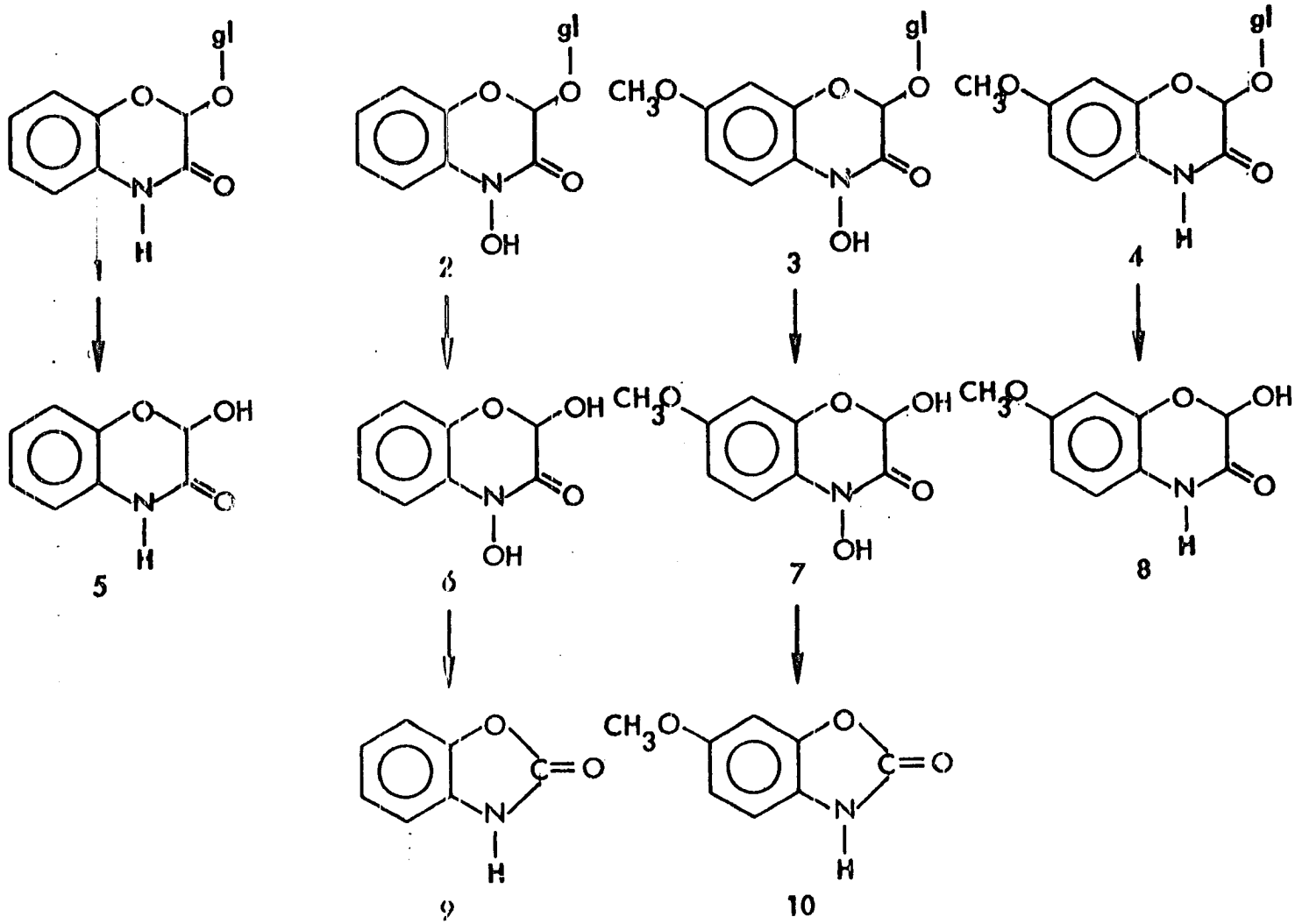
The two cyclic hydroxamic acids, DIMBOA and DIBOA, are the major ones occurring in higher plants. A new benzoxazolinone, 6,7-dimethoxy-2-benzoxazolinone, was isolated from dried tissue of corn (Zea mays L.) (18).

Approximately two dozen hydroxamic acids from fungi, yeast, and bacteria have been reported (19, 20). Suggested functions of these hydroxamic acids from microorganisms are as growth factors, antibiotics, antibiotic antagonists, tumor inhibitors, cell-division factors, and in iron metabolism (20). Page (21) has suggested that the hydroxamic acids may also have a function in iron metabolism in higher plants.

Resistance of corn to the European corn borer occurs at 3 stages of plant development. Most varieties of corn express resistance to the borer

Figure 1. Structure of cyclic hydroxamic acids and related compounds

1. HBOA-glucoside (2-hydroxy-1,4(2H)-benzoxazin-3-one-glucoisde)
2. DIBOA-glucoside (2,4-dihydroxy-1,4(2H)-benzoxazin-3-one-glucoside)
3. DIMBOA-glucoside (2,4-dihydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one-glucoside)
4. HMBOA-glucoside (2-hydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one-glucoside)
5. HBOA
6. DIBOA
7. DIMBOA
8. HMBOA
9. BOA (benzoxazolinone)
10. 6MBOA (6-methoxybenzoxazolinone)


gl
O
N
H
OH
CH3O
1
2
3
4
5
6
7
8
9
10

in the early seedling stage of plant development. At the whorl stage (about 30 - 33 in.extended leaf height) some varieties lose this resistance, and others retain it; resistance at this stage is termed 1st-brood borer resistance (22), since in the field susceptible plants at the whorl stage are normally attacked by the 1st-brood borer population. Resistance again occurs at the time when pollen is shedding (23). The antibiotic character of the resistance at this period is associated with the sheath and collar of the plant; such resistance has been termed 2nd-brood resistance (24, 25). Klun and Brindley (26) observed a linear relationship between the logarithm of the amount of 6MBOA isolated from various inbred varieties of maize at the whorl stage and the resistance rating to 1st-brood larvae of the European corn borer. The chemical basis of the resistance was identified by Klun et al. (27) based on the bioassay of DIMBOA in an artificial diet for the European corn borer. They found that DIMBOA inhibited larval development and caused 25% mortality.

When Beck and Stauffer (2) studied bioassay tests with the European corn borer and Penicillium to determine the activity of the resistance factor in tissue from the whorl leaf of inbred strains of dent corn, they found that the concentration of 6MBOA drops with the age of corn seedlings. Klun and Robinson (25) also observed that the concentration of DIMBOA in corn seedling plants drops as the corn grows. The rates of decrease in concentration of DIMBOA in 5 dent inbreds between 6 and 33 inches extended leaf height are in this order: CI31A~B49<OH43~B52«WF9. This is also the order of increasing susceptibility to corn borer attack at the whorl stage. Therefore, the loss of the borer resistance character by, e.g., WF9, between the seedling and whorl stages, is explained by the decrease of

DIMBOA concentration as the plant develops.

In addition to the resistance to attack by European corn borer larvae, Elnaghy and Linko (28) have suggested a correlation between cyclic hydroxamic acid content and stem rust resistance in wheat, while a similar correlation with resistance of maize varieties to stalk rot was suggested (29). Tolerance of certain plants to 2-chloro-s-triazine herbicides has also been related to the cyclic hydroxamic acids (30, 31). DIMBOA has also been shown to inhibit germination of spores of the phytopathogenic fungus Helminthosporium turcicum (32, 33).

The biochemical nature of the resistance of corn to the European corn borer has been studied extensively, but few biosynthetic studies of these resistance factors, benzoxazinones, have been carried out. Reimann and Byerrum reported the first study of the biosynthesis of DIMBOA in 1964 (34, 35). DIMBOA has two unusual structural features of biosynthetic interest. It contains a hydroxamic acid functional group and an oxazine ring moiety. Biosynthetic studies of other compounds containing a phenoxazine ring system found in insect pigments (ommochromes) (36) and fungus pigments (cinnabarin) (37, 38, 39) have shown that tryptophan is incorporated into these compounds. But the results of Reimann and Byerrum show that tryptophan is not incorporated into DIMBOA. They found that quinic acid-U-^{14}C, L-methionine-methyl-^{14}C, D-ribose-1-^{14}C, glycine-2-^{14}C, and glycerate-3-^{14}C are specifically and extensively incorporated into DIMBOA. The incorporation of D-ribose-1-^{14}C and glycerate-3-^{14}C into the aromatic ring of DIMBOA occurs via a 7-carbon sugar pathway and the shikimic acid pathway (40, 41). The evidence for the biosynthesis of the aromatic ring moiety of DIMBOA through shikimic acid is also provided by the

incorporation of quinic acid-U-^{14}C into the benzenoid ring carbons. The formation of aromatic compounds from quinic acid to shikimic acid in plants has been discussed in many reviews (42, 43, 44).

The 7-methoxy carbon of DIMBOA is formed by a transmethylation process from methionine. The biosynthesis of o-methyl groups in higher plants from methionine is well known (45).

Reimann and Byerrum (34, 35) observed that carbons 1 and 2 of ribose are incorporated into the two carbon atoms of the oxazine ring of DIMBOA. They suggested that the heterocyclic ring of DIMBOA has a biosynthetic pathway similar to that of the azine moiety of pteridines (46), and the pyrrole ring of tryptophan (47, 48, 49, 50). A nitrogen-containing aromatic compound in the shikimic acid pathway was suggested by them to condense with ribose, or a phosphorylated derivative, to form a ribosyl intermediate followed by an Amadori rearrangement, ring closure, and elimination of the triose moiety to give the oxazine ring. The postulated nitrogen-containing aromatic compound was recently demonstrated to be anthranilic acid by Tipton et al. (16).

Anthranilic acid appears to be the only primary aromatic amine produced directly from the shikimic acid pathway (44). It is a substrate for the production of biological materials other than tryptophan. In the leaves of Tecoma stans, anthranilic acid is hydroxylated to 3-hydroxyanthranilic acid by anthranilic acid hydroxylase (51) and two moles of this product are then converted by cinnabarinic acid synthetase to cinnabarinic acid via an oxidative dimerization reaction (52). Anthranilic acid is also oxidized to catechol via the intermediates of 3-hydroxyanthranilic acid and o-aminophenol by the multienzyme system of anthranilic acid

oxidase isolated from the same source (53). o-Aminophenol is also the intermediate between anthranilic acid and o-quinoneimine. The condensation of o-aminophenol and o-quinoneimine resulting in the biosynthesis of isophenoxazine is catalyzed by isophenoxazine synthetase (54). However, Tipton et al. (16) have shown from feeding experiments with maize that 3-hydroxyanthranilic acid and o-aminophenol are not incorporated into DIMBOA and are probably not intermediates. They reported that anthranilic acid is the intermediate.

The mechanism of formation of the N-hydroxyl groups is not clear. In studying the biosynthesis of hydroxamic acids of microbial origin it was observed that the hydroxylamine oxygen of hadicidin is derived from O_2 (55). N-hydroxylation reactions in mammalian liver are catalyzed by monooxygenases similar to those responsible for C-hydroxylation reactions (56, 57). It was also suggested that the hydroxamate moieties of aspergillic acid and mycelianamide are formed by oxidation of amide bonds (58, 59). Also the hydroxylation of the nitrogen in the amide group of 2-acetylaminofluorene catalyzed by an enzyme in rabbit liver microsomes was reported (60). Another possible mechanism for the formation of a cyclic hydroxamate, suggested by Emery (61), was that it could result from the reaction of a carboxyl compound with the free N-hydroxyamino acid. However, the biosynthesis of DIMBOA by a condensation reaction of ribose with an amine further suggests that the carbon-nitrogen bond is formed prior to the N-hydroxylation reaction.

An outstanding property of hydroxamic acids is the ability to form chelates with metal ions in stable five-membered rings (20, 62). A simple, useful qualitative analysis of hydroxamic acids depends, therefore, on the

intense red or blue color with ferric ion. Complexes with ferric ion occur in 3 steps: the complex is a 1:1 structure at low pH and transforms into a 3:1 complex as the pH approaches neutrality (20). Tipton and Buell (62) have determined the stability constants for the ferric ion complexes of DIMBOA and DIMBOA-glucoside from maize.

The herbicide simazine (2-chloro-4,6-bis-ethylamine-s-triazine) is used as a selective pre-emergence spray to control annual weeds in corn fields. The tolerance of corn to simazine was considered due to the detoxification of simazine to hydroxysimazine (2-hydroxy-4,6-bis-ethylamine-s-triazine) *in vivo* and *in vitro* by cyclic hydroxamic acids (63). Hydrolysis of simazine catalyzed by DIMBOA, not by its anion, has been suggested to be due to the molecular aggregates of the cyclic hydroxamic acid (64, 65).

Wahlroos and Virtanen (6) have presented the structure of 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) for the aglucone enzymatically formed in crushed rye seedlings. The structure was confirmed by the synthesis of the aglucone itself and its reduction product (13, 66). With the protection by a bulky group on the hydroxyl group of o-nitrophenol, DIBOA was synthesized with an overall yield of 3.5% by Honkanen and Virtanen (66). In their procedure, the starting material, o-methoxymethoxy-nitrobenzene, was reduced with zinc dust in ammonium chloride solution to form the corresponding phenylhydroxylamine derivative. The hydroxylamine was acetylated with dichloroacetyl chloride and the methoxymethoxy group was then hydrolyzed in acid. Finally, DIBOA was formed on further hydrolysis of the chlorine atoms in alkaline solution. They have also synthesized other 1,4-benzoxazine derivatives with the starting materials of

o-nitrophenoxy acetic acid ester (67) to study their antimicrobial activity. Although the synthesis of benzoxazines has been studied extensively (68), the procedures for the synthesis of DIBOA with higher yield and of DIMBOA have not been reported. Some cyclic hydroxamic acids have been synthesised successfully from the reduction of aromatic nitrocompounds by sodium borohydride catalyzed by palladium charcoal (69). A new synthesis of N-monosubstituted hydroxylamines from the reduction of oximes and nitro salts with diborane was reported (70, 71).

MATERIALS AND EXPERIMENTAL

Materials

Materials and instruments

Materials Maize seeds of the inbred varieties CI31A, WF9, B49, and B52 were obtained from Dr. W. A. Russell, Department of Agronomy, Iowa State University, Ames, Iowa. Vermiculite, W. R. Grace and Co., Cambridge, Mass.; silica gel GF_{254} (with fluorescent indicator), E. Merk, Darmstadt, Germany; Baker-flex silica gel IB-F (with fluorescent indicator), J. T. Baker Chemical Co., Phillipsburg, N. J.; UV lamp (254 nm, UVS-13), Brinkman Instruments Inc., Westbury, N. Y.; Sephadex G-25 Fine, Pharmacia Fine Chemical, Inc., Piscataway, N. J.; silicic acid (Brockman activity grade II), M. Woelm, Auswege, Germany; Eastman Kodak Blue X-ray film and Kodak No-Screen NS-2T X-ray film, Eastman Kodak Co., Rochester, N. Y.; Whatman glass fibre GF/A, W & R Balston Ltd., England; all compressed gases, Matheson Gas Products, Joliet, Ill.; quartz cuvettes, Hellma Cells, Inc., Jamaica, N. Y.

Instruments UV analyzer (UV optical unit ISCO Model UA and Model UA-2 UV analyzer), Instrumentation Specialties Co., Inc., Lincoln, Neb.; Cary 15 recording spectrophtometer (equipped with the Cary-Datex SDS-1 data recording system), Cary Instruments, Monrovia, Calif.; Beckman DU quartz spectrophotometer (optical density converter Model 205, light source Model 205, Gilford Instruments Laboratories, Oberlin, Ohio), Beckman Instruments Inc., South Pasadena, Calif.; Packard Model 3003 and 3310 Tri-Carb liquid scintillation spectrometers, and radiochromatogram

scanner Model 7201, Packard Instruments Co., Inc. La Grange, Ill.; Nuclear-Chicago gas flow counter (Model 1042 and 1048 planchet sample changers and Model 8703 series decade scalers), and Nuclear-Chicago Model 1620A analytical radioactive counter, Nuclear-Chicago Corporation, Des Plaines, Ill.; ATLAS mass spectrometer CH4, Varian Inc., Palo Alto, Calif.; high resolution mass spectrometer MS 902, AEI, Inc., Manchester, England.; Model R-20B high resolution nuclear magnetic resonance (NMR) spectrometer (60 MHz for hydrogen), Hitachi Perkin-Elmer, Hitachi, Ltd., Japan; Varian A-60 NMR spectrometer: plant growth chamber, Sherer Controlled Environment Lab., Sherer-Gillett Co., Marshall, Michigan.

Chemicals

For scintillator Naphthalene (analyzed reagent) and 1,4-dioxane (scintillation counting grade), J. T. Baker Chemical Co., Phillipsburg, N. J.; 2,5-diphenyloxazole (PPO) and 1,4-bix-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP), both scintillation grade, Packard Instrument Co., Inc., La Grange, Ill.

Biochemical compounds Adenosine-5'-triphosphate disodium salt (ATP), 5-phosphoribosyl-1-pyrophosphate magnesium salt (PRPP), D-glucose-6-phosphate monosodium salt (G-6-P), glucose-6-phosphate dehydrogenase (from Torula yeast, 1 unit oxidized 1 mmole of glucose-6-phosphate to 6-phosphogluconate), triphosphopyridine nucleotide monosodium salt (TPN), and p-nitrophenyl-β-D-glucose, Sigma Chemical Co., St. Louis, Mo.; ascorbic acid, Merk and Co., Inc., Rahway, N. J.; Folin-phenol reagent (2 N), Fisher Scientific Co., Fair Lawn, N. J.; phenylhydrazine hydrochloride (reagent grade), Eastman Kodak Co., Rochester, N. Y.

Radioactive chemicals D-ribose-1-^{14}C, L-ascorbic acid-1-^{14}C, and phenol-^{14}C, from New England Nuclear, Boston, Mass.; barium carbonate-^{14}C, Amersham/Searle Corporation, Arlington Heights, Ill.; simazine-^{14}C, Geigy Chemical Corporation, Ardsley, N. Y.; o-nitrotoluene-1-^{14}C, International Chemical and Nuclear Corporation, Irvine, Calif.

For chemical synthesis Palladium on powdered charcoal (10% catalyst), Matheson Coleman & Bell, Cincinnati, Ohio; ethyl chlorofluoroacetate, PCR Inc., Gainesville, Fla.; m-methoxyphenol and methyl dichloroacetate, Aldrich Chemical Co., Inc., Milwaukee, Wis.

Other chemicals were reagent grade unless otherwise specified.

General Methods

Culture of corn seedlings

Etiolated seedlings Maize seeds were sown in moist silica sand or vermiculite and incubated at 30°C in a dark chamber for 7 to 8 days with occasional watering. The etiolated seedlings (about 15 cm in height) were cut just above the sand or vermiculite and used for the experiments.

Green seedlings Maize seeds in moist vermiculite were incubated at 30°C in a plant growth chamber with automatic light control, 16 hours day and 8 hours night, for 7 or 8 days with occasional watering.

Isolation and purification of DIMBOA

The procedures described by Tipton et al. (14) were adopted. The

etiolated corn seedlings were chopped and ground with sea sand and a small amount of water with a mortar and pestle, or homogenized in a Waring Blender with water when a large number of seedlings was used. The homogenate was filtered through layers of cheesecloth or Pyrex wool, and the fibrous residue was squeezed to dryness. After standing for 30 minutes at room temperature to allow enzymatic hydrolysis of the glucosides, the filtrate was extracted three times with equal volumes of anhydrous ethyl ether in a 40 ml Pyrex centrifuge tube with a glass stopper. The aqueous solution and ether were mixed thoroughly and the emulsion was broken by centrifugation. The ether layer was transferred to a beaker by using a disposable Pasteur pipet and dried with anhydrous magnesium sulfate. After filtration, the ether was evaporated to dryness under vacuum on a rotary evaporator. The residue was washed with a minimum amount of chloroform: methanol (95:5, v/v) and the insoluble material was then dissolved in a minimum quantity of warm acetone. The acetone insoluble substance, if any, was discarded after centrifugation. Hexane (Skelly B) was added to the acetone solution until the solution became turbid. Upon standing for several hours, nearly pure white needle crystals of DIMBOA formed. Recrystallization in the same solvent gave a single $FeCl_3$ - positive spot on thin-layer chromatography in ether saturated with water.

Feeding of radioactive compounds to corn seedlings

Usually 5 etiolated 7- or 8-day-old seedlings were used for feeding experiments. The roots and seeds of the seedlings were cut off under water. The cut stems (about 15 cm high) were then dipped into a small volume of aqueous solution of the radioactive compound in a small beaker.

They were illuminated with one regular and one Gro-Lux fluorescent lamp, both 15 watts, at a distance of about 30 cm at room temperature in a hood. The radioactive solution was taken up by the seedlings through the cut stems in 4 or 5 hours, then another small volume of water was added until it was taken up again by the plants. More water was added, as needed, for the duration of the feeding. The plants were allowed to metabolize for a total of 24 hours. The seedlings were then homogenized and processed for the isolation of DIMBOA or further manipulations.

Thin-layer chromatography

Two different sizes of thin-layer chromatography (TLC) plates were used. Micro TLC was done on microscope slides (2.5 x 7.6 cm) coated with silica gel GF_{254} (20 slides per 6 g of silica gel in 12 ml of water). Other plates were made on 20 x 20 cm glass plates coated with silica gel GF_{254} to a thickness of 250 μ (5 plates per 30 g of silica gel in 60 ml of water). Baker-flex silica gel IB-F thin-layer chromatography sheets (20 x 20 cm and 5 x 20 cm) were also used. The chromatographic solvents used were: 1. ethyl ether saturated with deionized water: formic acid (99:1, v/v); 2. ethyl ether saturated with deionized water; 3. Benzene. Cyclic hydroxamic acids show a violet color when plates are developed in the first solvent. Benzoxazinones and other aromatic compounds were detected under a UV lamp in a dark room. Cyclic hydroxamic acids, DIMBOA and DIBOA, were also detected by spraying with acidic ferric chloride solution to give the blue color.

Acidic ferric chloride spraying reagent

$FeCl_3 \cdot 6H_2O$ (50 g) was dissolved in 500 ml of 0.1 N of hydrochloric acid in 95% ethanol.

Paper chromatography

A 20 x 20 cm Whatman No. 1 paper was used for the chromatography. The developing solvent of isoamyl alcohol saturated with 3 N HCl was used for ascending paper chromatography.

Column chromatography

Sephadex column Sephadex G-25 Fine (30 g) was swirled in 0.2 M phosphate buffer with pH 7.0 at room temperature for one day or in boiling buffer for 2 hours and packed into a 3 x 35 cm column.

Silicic acid column Silicic acid (200 g) was slurried with benzene and transferred to a 4 x 30 cm column and washed with the solvent.

Detection and measurement of radioactivity

Radioautography Radioactive compounds on thin-layer chromatograms or on a paper chromatogram were detected by exposure of these chromatograms with an Eastman Kodak Blue X-ray film or Kodak No-Screen NS-2T X-ray film in a lead foil lined holder. After one to two weeks of exposure, the X-ray film was developed.

Scintillation counting A Packard Model 3003 or 3310 Tri-Carb liquid scintillation spectrometer was used to measure radioactivity of radioactive samples in a scintillation glass vial with 15 ml of Bray's solution (72). The counting efficiency of the scintillation counter was calibrated to be

80% of dpm (disintegrations per minute) by the method reported by Baillie (73).

Detection of solid radioactive compounds Radioactivity of $BaCO_3$-^{14}C on Whatman glass fibre GF/A filter paper was measured with an automatic Nuclear-Chicago gas flow counter. The efficiency of this instrument was 30% of dpm. The carrier gas was 1.3% butane and 98.7% helium.

Specific radioactivity Specific radioactivity (S.A.) was expressed as counts per minute per micromole (cpm/μm) or dpm/μmole. The concentration of the radioactive sample was measured spectrophotometrically.

Feeding Experiments

Anthranilic acid-1-^{14}C

Anthranilic acid-1-^{14}C (5 μmole, specific activity 38,144 cpm/μmole) in 1.5 ml of water was fed to ten 7-day-old etiolated corn seedlings of CI31A (approximately 15 cm tall) through the cut stems. DIMBOA was crystallized from the ether extract. DIMBOA crystals were further degraded to 6MBOA by heating on a steam bath in a small volume of distilled water containing a few drops of pyridine for 2 hours (35). The aqueous solution was extracted with ethyl ether which was then evaporated to dryness. The degradation products were purified by thin-layer chromatography with silica gel GF_{254} (20 x 20 cm) developed in ether saturated with water. Two products were observed. 6MBOA was located at Rf = 0.80 and HMBOA was located at Rf = 0.50. HMBOA was considered to be one major impurity of DIMBOA during crystallization and it is fairly stable in boiling water. Both 6MBOA and HMBOA spots were scraped off the plates and transferred to

15 ml clinical centrifuge tubes and extracted with ethyl ether. The silica gel was removed by centrifugation. The solvent was evaporated to dryness. The concentration of each compound was measured in 95% ethanol spectrophotometrically and their specific radioactivities were determined, with a correction for the background from the TLC plates.

The crude ether extract of the corn seedlings and the degraded DIMBOA products were also chromatographed on thin-layer plates and then radioautographed.

After the corn seedling homogenate was extracted with ethyl ether, the protein was precipitated by trichloroacetic acid and dried by lyophylization. The total radioactivity of the protein was measured by scintillation counting.

o-Aminophenol-^{14}C

o-Aminophenol-^{14}C (5 µmole, specific radioactivity 24,956 cpm/µmole) in 1.5 ml of water was fed to ten 7-day-old etiolated corn seedlings of CI31A for 24 hours. DIMBOA was isolated and crystallized and then degraded to 6MBOA. A radioautograph was made and the specific activity of 6MBOA was measured.

o-Aminophenol-^{14}C + phenylhydrazine + ascorbic acid

o-Aminopheno (2.5 µmole, specific activity 31,491 cpm/µmole), 0.5 µmole of phenylhydrazine hydrochloride and 10 umole of ascorbic acid in 2 ml of 0.02 M phosphate buffer, pH 7.0, were fed to five 7-day-old etiolated corn seedlings of CI31A for 24 hours. DIMBOA was crystallized

and degraded to 6MBOA. Specific activity of 6MBOA was measured.

Anthranilic acid-1-^{14}C + phenylhydrazine + ascorbic acid

A 2 ml of 0.02 M phosphate buffer (pH 7.0) solution containing 2.5 µmole of anthranilic acid-1-^{14}C (specific activity 58,511 cpm/µmole), 0.5 µmole of phenylhydrazine, and 10 µmole of ascorbic acid was fed to the cut stems of five 7-day-old etiolated corn seedlings of CI31A. DIMBOA was crystallized after 24 hours feeding and then degraded to 6MBOA. The specific activity of 6MBOA was measured.

D-ribose-1-^{14}C

An aqueous solution (5 ml) of 1.8 µmole of D-ribose-1-^{14}C (specific activity 9.77×10^6 cpm/µmole) was fed to the cut stems of fifty 7-day-old etiolated corn seedlings of CI31A for 24 hours. DIMBOA was crystallized and its specific activity was measured. DIMBOA crystals were also used for the study of the specific carbon-14 labeling in the DIMBOA molecule which will be described later. Radiochemical purity of the commercial D-ribose-1-^{14}C was approved by paper chromatography using N-butanol: pyridine: water (6:4:3, v/v) as the solvent system. Radioactivity on the paper chromatogram was detected with a radiochromatogram scanner. Scan speed was 1 cm/min, 30 seconds time constant, collimator width 2mm. The carrier gas was 0.9% isobutane and 99.0% helium.

L-ascorbic acid-1-^{14}C

Several concentrations of L-ascorbic acid-1-^{14}C (specific activity 6.00×10^6 cpm/µmole) in small volumes of water were fed to groups of 10

to 50 etiolated corn seedlings of CI31A (7-day-old) for 24 hours. The specific activity of DIMBOA was measured and the crystals of DIMBOA were also used for the study of the specific carbon-14 labeling in the DIMBOA molecule.

Degradation of DIMBOA

The procedure for degradation of DIMBOA described by Reimann and Byerrum (35) was used to determine the specific carbon atoms or groups labeled by the isotope within the molecule of DIMBOA. Some of the procedures have been modified in this laboratory. The degradation procedure devised permitted the isolation of carbons in position 2 and 3 as shown in Figure 2.

Conversion of DIMBOA to 6MBOA

About 20 mg of radioactive DIMBOA in 3 ml of H_2O and 0.1 ml of pyridine were refluxed for 3 hours. The solution was then distilled under reduced pressure into a receiving flask cooled in an ice bath. 6MBOA was in the residue and formic acid liberated from C-2 of the molecule was in the distillate.

The amorphous residue, obtained after the distillation of the solvents from the reaction mixture, was crystallized from hot water to yield reddish-tan needles of 6MBOA.

Conversion of HCOOH to $BaCO_3$

The formic acid solution obtained by distillation was acidified to

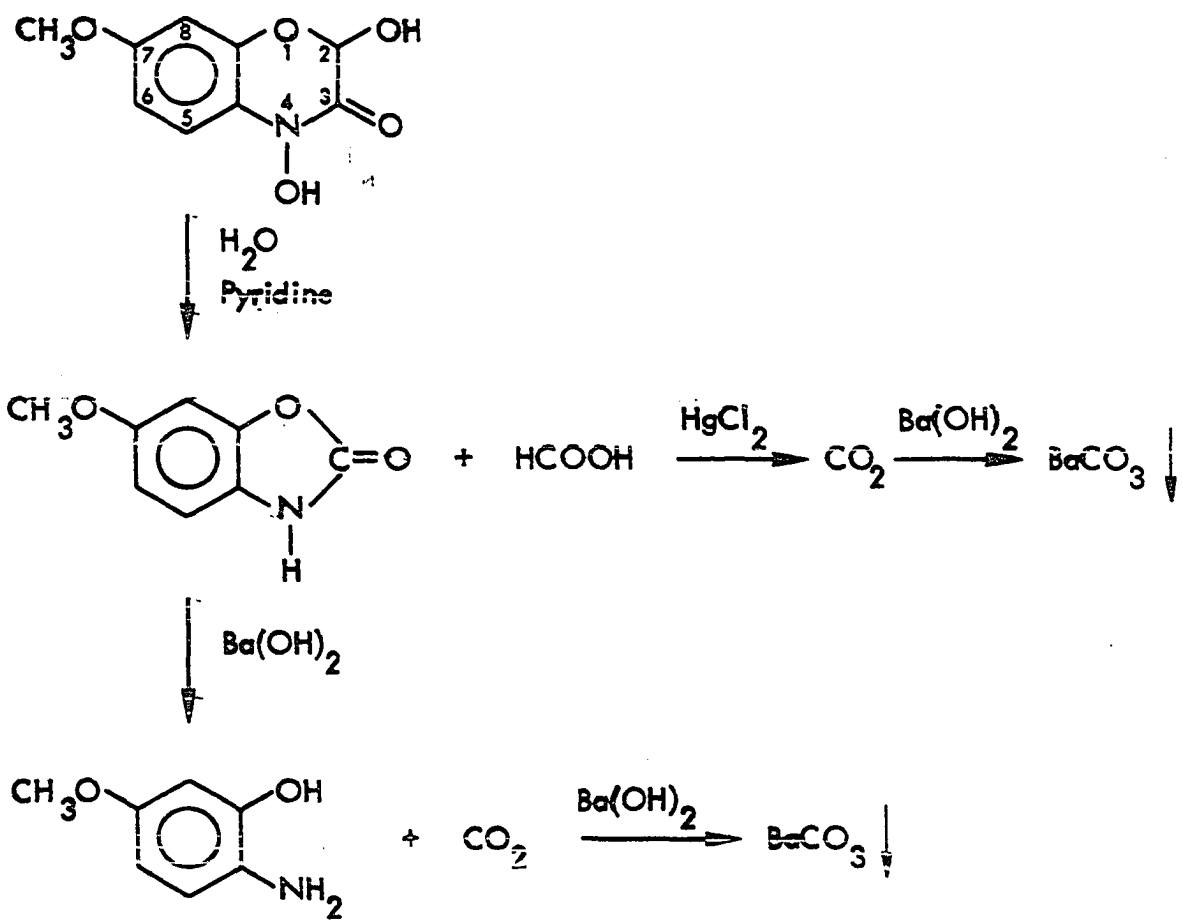


Figure 2. The degradation procedure for DIMBOA

pH_3 with concentrated hydrochloric acid. According to the procedure of Sakami (74), 2 ml of CO_2-free mercuric chloride in acetate buffer solution was added to the formic acid solution and boiled for one hour under nitrogen. CO_2 liberated from formic acid was driven by nitrogen to a flask containing saturated barium hydroxide solution. Barium carbonate

was filtered with Whatman glass fibre GF/A filter paper, washed with water, ethanol, and ether, and dried in the desiccator under vacuum. Specific radioactivity of the $BaCO_3$-^{14}C was determined by Nuclear-Chicago gas flow counter.

Degradation of 6MBOA

About 50 μmole of 6MBOA (8.3 mg) was refluxed in 20 ml of saturated barium hydroxide under nitrogen overnight. The reaction flask was covered with aluminum foil. An additional barium hydroxide solution was connected between the reaction flask and the nitrogen tank to remove traces of carbon dioxide in the nitrogen gas. Barium carbonate was formed in the reaction flask and was then decomposed by injection of concentrated hydrochloric acid. Carbon dioxide liberated was collected in another fresh barium hydroxide solution. After one hour, barium carbonate was filtered and its specific radioactivity was measured as described before.

After degradation of 6MBOA, the solution in the reaction flask contained 5-methoxy-o-aminophenol and barium salts. The solution was neutralized with sodium hydroxide solution and extracted with ethyl ether. The ether extract shows one spot at Rf = 0.72 on silica gel GF_{254} TLC in ether saturated with water. The Rf value was close to the standard value (0.73). The specific activity was determined by scintillation counting.

Experiments with Cell Free Extracts of Corn Seedlings and Radioactive Precursors of DIMBOA

Preparation of extracts

The procedures described by Tu (75) were adopted. Thirty grams of

7-day-old etiolated corn seedlings of the inbred variety CI31A was chopped and homogenized for two minutes with 30 ml of 0.02 M potassium phosphate buffer, pH 7.0, containing 0.01 M potassium ascorbate, 0.01 M mercaptoethanol, and 0.001 M magnesium chloride, in an ice-jacketed Waring Blender. The homogenate was filtered through layers of cheesecloth and designated E-1.

Fifteen grams of 7-day-old etiolated corn seedlings of CI31A was homogenized with 15 ml of 0.2 M potassium phosphate buffer, pH 7.0, in an ice-jacketed blender for 2 min and the homogenate was filtered through layers of cheesecloth at cold room (4°C). The filtrate was transferred to the Sephadex G-25 Fine column and eluted with the same buffer solution at 4°C at a flow rate of 2 ml/min. The protein fraction was collected at the first UV absorption peak, which came out at 30 ml of eluate and was designated E-2.

The etiolated corn seedlings (100 g 7-day-old) was homogenized with cold acetone (-18°C) two times in a Waring Blender. Each time the homogenate was filtered through a large Buchner funnel. The residues were dried in a desiccator under vacuum in the cold room overnight. One gram of acetone powder was homogenized with 15 ml of 0.02 M phosphate buffer, pH 7.0, (containing 0.01 M potassium ascorbate, 0.01 M mercaptoethanol, and 0.001 M chloride), pH 7.0. The filtrate was designated as E-3.

Anthranilic acid-1-^{14}C and o-aminophenol-^{14}C with extracts

A 2.5 ml reaction mixture contained: anthranilic acid-1-^{14}C, 1 μmole (specific activity 49,498 cpm/μmole) or 1 μmole of o-aminophenol-^{14}C (specific activity 24,956 cpm/μmole); ATP, 2 μmoles; TPN, 2 μmoles;

PRPP, 2 μmoles; glucose-6-phosphate, 2 μmoles; glucose-6-phosphate dehydrogenase, 2 units; and 1.9 ml of corn extract E-1 or E-2, or E-3. In the reaction with E-2, ascorbate, mercaptoethanol, and magnesium were added at the same concentrations as in E-1 or E-3. The reaction was carried out at 30°C and stopped by mixing with an equal volume of cold ethyl ether after specified periods. Boiled corn extract and the reaction mixture of substrates and cofactors were used as control. The ether extract was dried and redissolved in 0.1 ml of 95% ethanol a portion (0.01 ml) of this solution was spotted on a 20 x 20 cm. Baker-flex silica gel IB-F TLC plate and developed in ether saturated with water. The aqueous solution after ether extraction was lyophylized and redissolved in 0.1 ml of water. Aliquots (0.01 ml) of the solution was spotted on a 20 x 20 cm paper chromatogram and developed in 80% ethanol. Radioactive substances on thin-layer and paper chromatograms were detected by radioautography.

Fixation of Radioactive CO_2 into Corn Seedlings

Twenty five light-grown corn seedlings in vermiculite (10-day-old) were placed in a sealed glass chamber which was connected to a CO_2 generator at one end and to an analytical radioactive counter Model 1620 A (Nuclear-Chicago) at other end. The chamber was illuminated with 6 regular fluorescent lamps (each one 20 watts) at a distance of about 40 cm and was at room temperature. A peristaltic circulating pump (Schaas and Co., Chicago, Ill.) was connected between the CO_2 generator and the radioactive counter to circulate the gas in the whole completely sealed system. The radioactive $BaCO_3$ (100 μmole, total activity 251 x 10^6 dpm) was placed in the generator. It was then decomposed by injection of 5 ml of lactic acid

and the solution was kept slightly warm to drive the CO_2 from the solution. The radioactivity of the circulating CO_2 was measured in cpm by the counter. After 4 hours for radioactive CO_2 fixation, the unused radioactive CO_2 was removed by passing the air in the chamber through a $Ba(OH)_2$ receiver which was blocked during fixation. At specified time intervals after this, 2 seedlings at a time were cut and removed from the chamber, weighed, and homogenized with small volume of water in a mortar. The homogenate was extracted with ethyl ether in a 40 ml Pyrex centrifage tube. The ether extract was evaporated to dryness. DIMBOA was then separated from the pigments by Baker-flex silica gel IB-F TLC in ether saturated with water. The partially purified DIMBOA was decomposed in water in a sealed vial at 80°C for several hours. The aqueous solution was extracted again with ethyl ether. The ether was evaporated and the residue wad dissolved in 0.1 ml of 95% ethanol. Aliquots of 0.95 ml were spotted on the same kind of TLC for the purification of 6MBOA and HMBOA. The concentrations of 6MBOA and HMBOA were measured spectrophotometrically and their specific activities were measured in 1,4-dioxane scintillator by Packard Tri-Carb spectrometer.

Enzymatic Reduction of HBOA and the Activity of β-glucosidase

Assay of 2-hydroxy-benzoxazinone reductase activity

HBOA was reduced by 2-hydroxy-benzoxazinone reductase to give N-glycolyl-o-aminophenol (GAP) (17). The procedure for the assay of the enzyme was described by Wang (17). A total of 2.5 ml of reaction mixture contained: 1 μmole of HBOA; 2 μmoles of TPN; 2 μmoles of glucose-6-phosphate; 2 units of glucose-6-phosphate dehydrogenase; and 2 ml. of the

protein fraction E-2. Phosphate buffer (0.2 M, pH 7.0) was used to make up the volume to 2.5 ml. The reaction mixture was incubated at 37°C for a specified period. The reaction was stopped by cooling and extracting three times with 2.5 ml volumes of ethyl ether. The ether was evaporated to dryness and the residues were dissolved in 0.1 ml of 95% ethanol. The solution (0.07 ml) was spotted on (silica gel GF_{254}) TLC which was then developed in ether saturated with water. HBOA has an Rf value of 0.50 and the product (GAP) has an Rf value of 0.43 in this system. The product spot was scraped from the TLC plate and transferred to a 15 ml clinical centrifuge tube. GAP was extracted with ethyl ether and the silica gel was removed by centrifugation in a clinical centrifuge at maximum speed for 15 min. The supernatant was evaporated to dryness and redissolved in 3 ml of 95% ethanol. The quantity of GAP was measured spectrophotometrically.

A reaction mixture containing HBOA, TPN and the protein fraction E-2 was also investigated under the same conditions as above.

Assay of β-glucosidase

The procedure described for the determination of phosphomonoesterase in serum with p-nitrophenylphosphate (76) was applied to measure the activity of β-glucosidase in corn plants. A 2 ml of the protein fraction of E-1 (pH 7.0) was diluted to 10 ml with 0.2 M acetate buffer, pH 5.2. The pH of the diluted protein solution was only increased slightly to 5.4. This diluted enzyme solution was used for the assay. The reaction mixture containing 1 ml of acetate buffer, 1 ml of the substrate, p-nitrophenyl-D-glucoside (1 mg/ml) and 0.25 ml enzyme solution was incubated at

30°C for a specified period. The reaction was stopped by adding 5 ml of 0.1 N NaOH solution. The optical density was measured at 410 nm with a Beckman DU spectrophotometer.

Determination of Proteins in cell-free extracts of corn seedlings

A method of estimate the protein content in plant tissues containing phenolic materials was developed by Potty (77). Three reagents were first prepared: reagent A. 2% sodium carbonate in 0.1 N NaOH solution; reagent B. Alkaline copper reagent, prepared by mixing 1 ml of a solution containing 0.5% copper sulfate in 1% sodium tartrate with 50 ml of reagent A. Reagent C. Folin phenol reagent was diluted to a final acidity of 1 N. In the procedure, 1 ml aliquots containing 100 to 400 ug of proteins were mixed with 5 ml each of reagents A and B in separate tubes. A portion (1 ml) of reagent C was added to each after 10 min. After standing at room temperature for 30 min, the optical density was measured at 500 nm with a Beckman DU Spectrophotometer. The difference between the two readings was used to calculate the protein from a reference graph prepared using standard ovalbumin in the above procedure.

Detoxification of Simazine by DIMBOA in vitro

The detoxification of simazine by DIMBOA was carried out using 20 mumoles of ring labelled simazine-^{14}C (specific activity 3.46×10^6 dpm/ umole) and 0, 0.4, and 4 umoles of DIMBOA in 2 ml of citrate buffer, pH 4.8. Aliquots of these solutions (0.2 ml) were transferred to 15-ml vials and incubated at 37°C. At intervals of 1 hour, vials containing the higher concentration of DIMBOA were removed and lyophilized. At intervals

of 10 hours, vials containing the lower concentration of DIMBOA and controls without DIMBOA were removed and lyophilized. The residues were dissolved in $CHCl_3$: CH_3OH : H_2O (4:4:1, v/v) without heating and transferred to sheets of Whatman No. 1 paper, developed in isoamyl alcohol saturated with 3 N HCl and the radioactive spots counted.

Degradation of DIMBOA in Hydrochloric Acid Solution

DIMBOA (4.7 μmoles) was dissolved in 100 ml of 1 N HCl. The solution was incubated at 37°C. About 3 ml of the solution was removed after specified periods and cooled in dry ice-acetone for few seconds. The UV spectrum of the solution was measured by Cary 15 equipped with the Cary-Datex SD-1 data recorder. In order to determine the structure of the degradation products in the hydrochloric acid solution, a large quantity of the reaction solution was incubated at 80°C for 3 days to let the reaction be completed. The acidic solution was neutralized with sodium hydroxide solution and extracted with ethyl ether. The ether solution was dried with anhydrous magnesium sulfate, filtered, and evaporated to a small volume. The products were purified by silica gel GF_{254} TLC developed in the solvent system: chloroform: ether saturated with water: 95% ethanol (4:1:1, v/v). The structures of the products were inferred from their UV spectra and mass spectra.

Chemical Synthesis

o-Aminophenol-^{14}C and anthranilic acid-1-^{14}C were synthesized by the procedures reported by TU (75). o-Aminophenol-^{14}C was synthesized by the nitration of phenol-^{14}C. The o-nitrophenol-^{14}C was crystallized in

water and the wet crystals were hydrogenated. The product was purified by sublimation to give white needles with m.p. 171°C. The specific activity of o-aminophenol-^{14}C was 3.15×10^4 cpm/μmole.

Anthranilic acid-1-^{14}C was synthesized by the oxidation of o-nitrotoluene-1-^{14}C followed by hydrogenation. The synthetic anthranilic acid-1-^{14}C showed one fluorescent spot on silica gel GF_{254} TLC when it was exposed UV light. Its specific activity was 5.85×10^4 cpm/μmole and it melted at 145°C.

Synthesis of DIBOA

Preparation of potassium o-nitrophenolate o-Nitrophenol (13.9 g, 0.1 mole) was dissolved in 50 ml absolute ethanol with gentle warming; 5.6 g of potassium hydroxide (0.1 mole) was dissolved in 200 ml of absolute ethanol also with gentle warming. The potassium hydroxide solution was filtered into a one-liter beaker and the o-nitrophenol solution was added with stirring. Orange needles formed in one hour and were filtered after 2 hours, washed with 2 x 200 ml portions of absolute ethanol and 3 x 200 ml of anhydrous ethyl ether, dried in air. The yield was 100%.

Preparation of ethyl o-nitrophenoxy fluoroacetate To 5 g of potassium o-nitrophenolate (0.028 mole) in 100 ml of dimethylformamide was added 5 g of ethyl chlorofluoroacetate. The solution was stirred for 30 min and stood for 2 days at room temperature, during which a white precipitate of potassium chloride formed. The reaction mixture was then diluted with 100 ml of cold water and extracted with 3 portions (200 ml each) of ethyl ether. The pooled ether solutions were washed with 3 portions (50 ml each) of water, dried with anhydrous magnesium sulfate, filtered and

the solvent removed under vacuum with a rotary evaporator at 20-25°C. The resulting yellow oil was applied to a column of silicic acid, (200 g in 4 x 30 cm), which was then eluted with benzene. After a yellow band (o-nitrophenol) was eluted, an additional 600 ml benzene was used to elute the product. The benzene was removed under vacuum at 30°C in a rotary evaporator yielding a light yellow oil in 75% yield. Boiling point: decomposes at 120°C. Thin layer chromatography; Rf = 0.45 on silica gel GF_{254} developed with benzene. The UV and mass spectra of this compound were measured.

Preparation of 2,4-dihydroxy-1,4(2H)-benzoxazine-3-one To a 200 ml of water was added 1 g of ethyl o-nitrophenoxyfluoroacetate and the mixture was stirred at room temperature. A solution of ammonium chloride (1 g in about 10 ml water) and 1.5 g of zinc dust were added to the aqueous mixture of ethyl o-nitrophenoxyfluoroacetate over a period of 15 min. The mixture was then filtered and the zinc dust was returned to the reaction beaker. The walls of the beaker were washed down with a small amount of ethyl ether and another portion of ammonium chloride solution (1 g in 200 ml water) was added. After stirring 15 minutes the reaction mixture was again filtered. The filtrates were combined, and ethylenediaminetetraacetate acid (EDTA) was added to saturate the solution. After 5 minutes stirring the solution was extracted with several portions of ethyl ether. The combined ether extracts were washed with two small portions of water and dried with anhydrous magnesium sulfate, filtered and evaporated to the smallest volume in vacuum at room temperature with a rotary evaporator (no crystals formed after evaporation of ether). The brown gummy product was crystallized in acetone-benzene and the white needle crystals were washed

with a small volume of benzene. The yield was 30%. The overall yield of DIBOA was 22%. The product melts at 147-8°C (lit. 151-3°C). Its UV and mass spectra were measured. It had an Rf value of 0.48 on silica gel GF_{254} developed in ether saturated with water and showed a blue color after spraying with $FeCl_3$ solution.

Miscellaneous reactions

Preparation of methyl bis-(o-nitrophenoxy)-acetate The reaction of potassium o-nitrophenolate (1 g) and methyl dichloroacetate (1 g) was carried out in 20 ml of dimethylformamide at 80°C for 3-5 hours with stirring. The product was purified by the same procedure as described for ethyl o-nitrophenoxyfluoroacetate. After silicic acid column purification, the product was recrystallized in benzene-Skelly B (hexane) and gave light yellow needles which melt at 90°C. The Rf value on silica gel GF_{254} developed with benzene was 0.22. The NMR spectrum and mass spectrum were measured. The yield was 70%.

Preparation of 2-o-aminophenoxy-4-hydroxy-1,4(2H)-benzoxazin-3-one (AHROA) Methyl bis-(o-nitrophenoxy) acetate (100 mg) was reduced in 20 ml of 80% ethanol with ammonium chloride (100 mg) and zinc dust (150 mg) within 30 minutes at room temperature. After adding a small volume of water, the reduced product was extracted with several portions of ethyl ether. The ether solution was washed with a small volume of water and dried with anhydrous magnesium sulfate, filtered and evaporated to dryness in vacuum at room temperature with rotary evaporator. The solid resolve was recrystallized from acetone-Skelly B (hexane) and the mass and UV spectra were measured. The zinc concentration of this reduced product was

analyzed by Analytical Chemistry Group I, Ames Laboratory Atomic Energy Commission, Ames, Iowa. This compound melts at 156-158°C and has Rf = 0.56 on silica gel GF_{254} developed in ether saturated with water. It also shows a positive reaction with $FeCl_3$ spraying reagent. The yield was 80%. The overall yield of the reduced compound was 56%.

RESULTS

Metabolism of Benzoxazinones

Feeding Experiments

The efficiency of incorporation of radioactivity into DIMBOA or its degradation products is expressed in terms of the dilution of specific activity (S. A.), i.e., the specific activity of substrate divided by the specific activity of DIMBOA or its degradation products. The results of the feeding experiments are summarized in Table 1.

These experiments can be divided into three categories: the incorporation of anthranilic acid-1-^{14}C and o-aminophenol-^{14}C; feeding of these two substrates accompanied by phenylhydrazine and ascorbic acid; and the distribution of ^{14}C in the DIMBOA molecule from labeled D-ribose-1-^{14}C and L-ascorbic acid-1-^{14}C.

The feeding of anthranilic acid-1-^{14}C showed the incorporation of this compound into DIMBOA and HMBOA. The specific activity and dilution factor for DIMBOA were measured from its degradation product 6MBOA. There was no incorporation of o-aminophenol-^{14}C into DIMBOA even when phenylhydrazine and ascorbic acid were added in the feeding solution. The feeding of anthranilic acid-1-^{14}C, phenylhydrazine and ascorbic acid was used as a control for experiment No. 4 in Table 1 and resulted in the same dilution factor as from the feeding of anthranilic acid-1-^{14}C alone.

L-ascorbic acid-1-^{14}C was incorporated into DIMBOA. The dilution factor changed when the concentration of L-ascorbic acid-1-^{14}C and the number of corn seedlings used varied. A high concentration of the substrate

Table 1. The incorporation of radioactive substrates into DIMBOA and the distribution of ^{14}C in the DIMBOA molecule from labeled precursors

No.	Substrates	S.A. (cpm/μmole)	Amount (μmole)	Number of seedlings	DIMBOA S.A. (1) (cpm/μmole)	DIMBOA Dilution factor
1.	Anthranilic acid-1-^{14}C	3.81×10^4	5.0	10		
2.	o-Aminophenol-^{14}C	2.50×10^4	5.0	10		
3.	Anthranilic acid-1-^{14}C Phenylhydrazine L-ascorbic acid	5.85×10^4	2.5 0.5 10.0	5		
4.	o-Aminophenol-^{14}C Phenylhydrazine L-ascorbic acid	3.15×10^4	2.5 0.5 10.0	5		
5.	D-ribose-1-^{14}C	9.77×10^6	1.80	50	1058	9.2×10^3
6.	L-ascorbic acid-1-^{14}C	4.97×10^6	1.80	50	34	1.5×10^5
7.	L-ascorbic acid-1-^{14}C	6.00×10^6	1.48	10	1250	4.8×10^3
8.	L-ascorbic acid-1-^{14}C	6.00×10^6	1.48	50	111	5.4×10^4
9.	L-ascorbic acid-1-^{14}C	6.00×10^6	2.96	50	419	1.4×10^4

Table 1. (Continued)

6MBOA			$BaCO_3$ (C-3 of DIMBOA)		$BaCO_3$ (C-2 of DIMBOA)		HMBOA	
S.A. (2) (cpm/μmole)	Dilution factor	$\frac{\text{S.A. (2)}}{\text{S.A. (1)}}$ %	S.A. (3) cpm/μmole	$\frac{\text{S.A. (3)}}{\text{S.A. (2)}}$ %	S.A. (4) cpm/μmole	$\frac{\text{S.A. (4)}}{\text{S.A. (1)}}$ %	S.A. cpm/μmole	Dilution factor
779	49						378	101
0								
1216	48							
0								
1077		102	417	39.4	63	5.8		
54		114	0.8	1.5	2.5	4.5		
151		136	13	8.6				
390		94			10.2	2.6		

and a smaller number of seedlings resulted in better incorporation. The feedings of D-ribose-1-^{14}C and L-ascorbic acid-1-^{14}C were under the same conditions, i.e., both substrates were at the same concentration and the same number, same age of corn seedlings was used. In Table 1, the results show that the dilution of L-ascorbic acid-1-^{14}C is 16 times higher than that of D-ribose-1-^{14}C, i.e., D-ribose-1-^{14}C has better incorporation than L-ascorbic acid-1-^{14}C into DIMBOA.

Results of the degradation of DIMBOA show that the incorporation of D-ribose-1-^{14}C was 40% at the C-3 atom in the DIMBOA molecule and only 6% at C-2 atom. L-ascorbic acid-1-^{14}C was incorporated in a more nearly random manner.

The radioautograph of products of the feeding of anthranilic acid-1-^{14}C is shown in Figure 3. Figure 3-A is the radioautograph of the ether extract of the homogenate of corn seedlings. The radioactive compounds are: DIMBOA at Rf 0.62; HBOA at Rf 0.71; 6MBOA at Rf 0.78; and BOA at Rf 0.83. Figure 3-B represents the results from the degradation of DIMBOA in water. The spots at Rf 0.81 and 0.59 are 6MBOA and HMBOA, respectively.

The total radioactivity of the protein from plants fed anthranilic acid-1-^{14}C was only 0.1% of the total radioactivity of the ether extract.

Experiments with Cell-Free Extracts of Corn Seedlings and Radioactive and Nonradioactive Precursors of DIMBOA

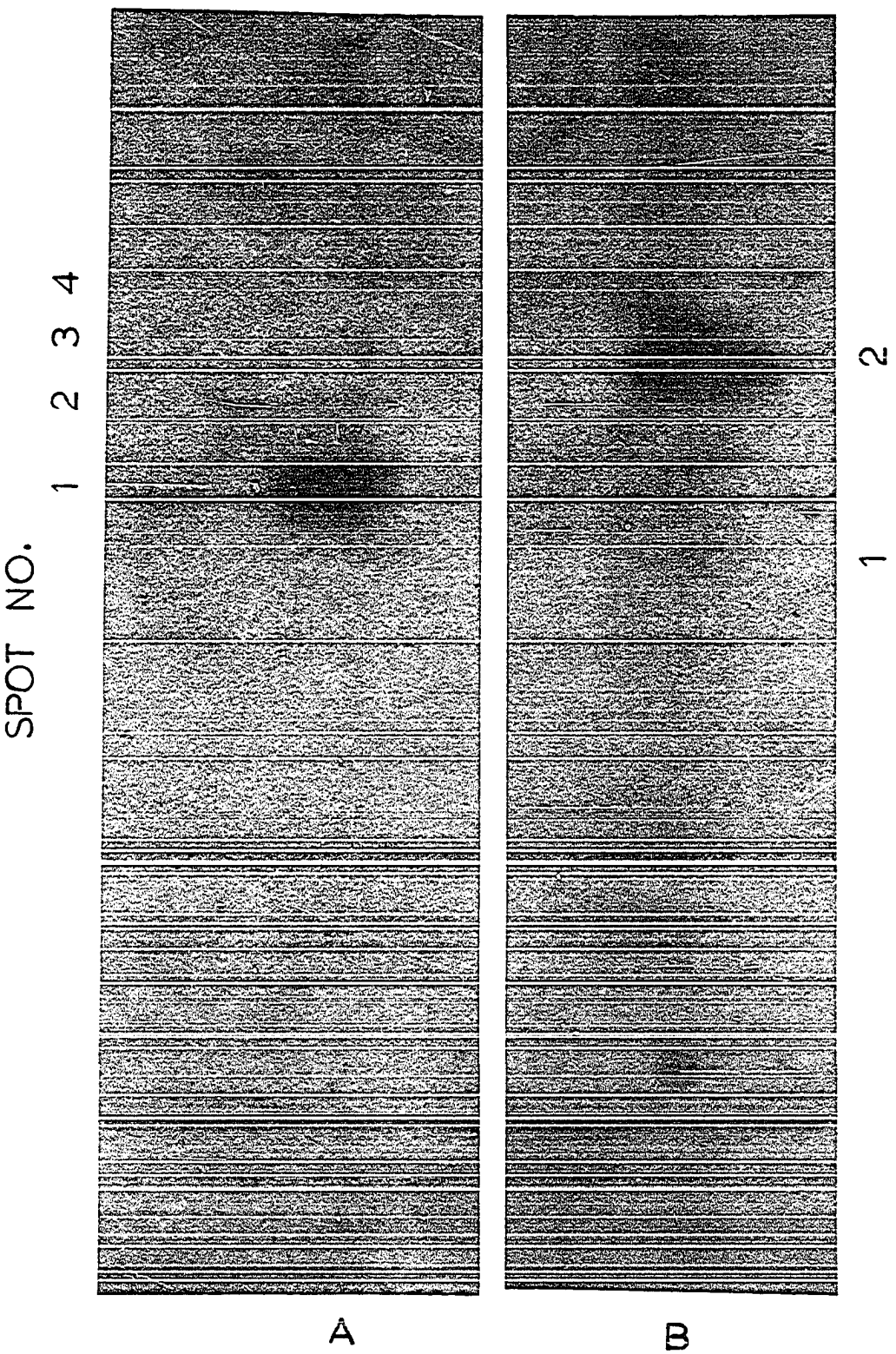
Anthranilic acid and the cell free extract E-1

A radioautogram of a thin-layer chromatogram of the ether-soluble products resulting from incubation of anthranilic acid-1-^{14}C with a cell-

Figure 3. Radioautograph of Baker-flex silica gel IB-F TLC developed in ether saturated with water.

A. Representing the ether-soluble materials from corn seedlings fed anthranilic acid-1-^{14}C
Rf values: Spot No. 1 = 0.62, DIMBOA and HMBOA; spot No. 2 = 0.71, HBOA; spot No. 3 = 0.78, 6MBOA; spot No. 4 = 0.83, BOA.

B. Representing the degradation of the product at Rf = 0.62 in water
Rf values: Spot No. 1 = 0.59, HMBOA; spot No. 2 = 0.81, 6MBOA.


SPOT NO.
1 2 3 4
A
B
1 2

free extract of seedling leaves (Figure 4-A) shows several labeled products but no incorporation of ^{14}C into DIMBOA. Even at zero time of the reaction, 3 radioactive spots (spots 1, 2, and 4) were observed in addition to the substrate anthranilic acid-1-^{14}C (spot 7). These 3 radioactive products have not been identified. The concentration of spot No. 4 decreases with increasing incubating time. Figure 4-B shows the radioautogram of the ether-soluble materials after degradation in water and again shows no labeling in either 6MBOA or HMBOA.

Spots 1 and 3, Figure 4-B, are the same compounds as spots 2 and 4 in Figure 4-A, respectively. Figures 4-Aa and 4-Bb represent the thin-layer chromatograms corresponding to Figures 4-A and 4-B, respectively. The S.A. of 6MBOA and HMBOA, obtained by eluting these compounds from the thin-layer plate shown in Figure 4-Bb, were found to be 5 and 97 cpm/umole, respectively. The S.A. of 6MBOA is negligibly low and the radioactivity found in HMBOA is probably due to contamination by the material in spot 1, Figure 4-B, which has an Rf value close to that of HMBOA. Again there is no evidence for incorporation of anthranilic acid-1-^{14}C into DIMBOA in the cell free extract. The aqueous solution after ether extraction has no significant radioactivity.

Control experiments show that the material in spot 4 in Figure 4-A is a product of nonenzymatic reaction of anthranilic acid and DIMBOA. Figure 5 is the thin-layer chromatogram of the controls. Reaction I has no product. It is probably due to the concentration of DIMBOA in E-1 diluted 1:10 with buffer is too small. Reaction II is the control to check the stability of anthranilic acid. Reactions of number III and IV show that the product at Rf = 0.68 is the same compound as unknown a in

Figure 4. Radioautograph of the ether soluble materials from anthranilic acid-1-^{14}C and cell-free extract E-1.

TLC: Baker-flex silica gel IB-F developed in ether saturated with water

A. Anthranilic acid-1-^{14}C, 1 µmole; ATP, 2 µmoles; PRPP, 2 µmoles; TPN, 2 µmoles; G-6-P, 2 µmoles; G-6-P dehydrogenase, 2 units; and 1.9 ml E-1 at 30°C; total volume, 2.5 ml in phosphate buffer (pH 7.0); time of reactions: I. 0; II. 5 min; III. 15 min; IV. 30 min

Spot No. 1. unknown c; 2. unknown b; 3. DIMBOA; 4. unknown a; 5. HBOA; 6. unknown; 7. anthranilic acid-1-^{14}C; (Spot No. 3, 5, and 6 are shown in Figure 4-Aa)

B. Ether-soluble materials from A-II after degradation in water

Spot No. 1. unknown b; 2. HMBOA; 3. unknown a; 4. HBOA; 5. 6MBOA; 6. anthranilic acid-1-^{14}C; 7. impurity of anthranilic acid-1-^{14}C (Spot No. 2, 4, and 5 are shown in Figure 4-Bb)

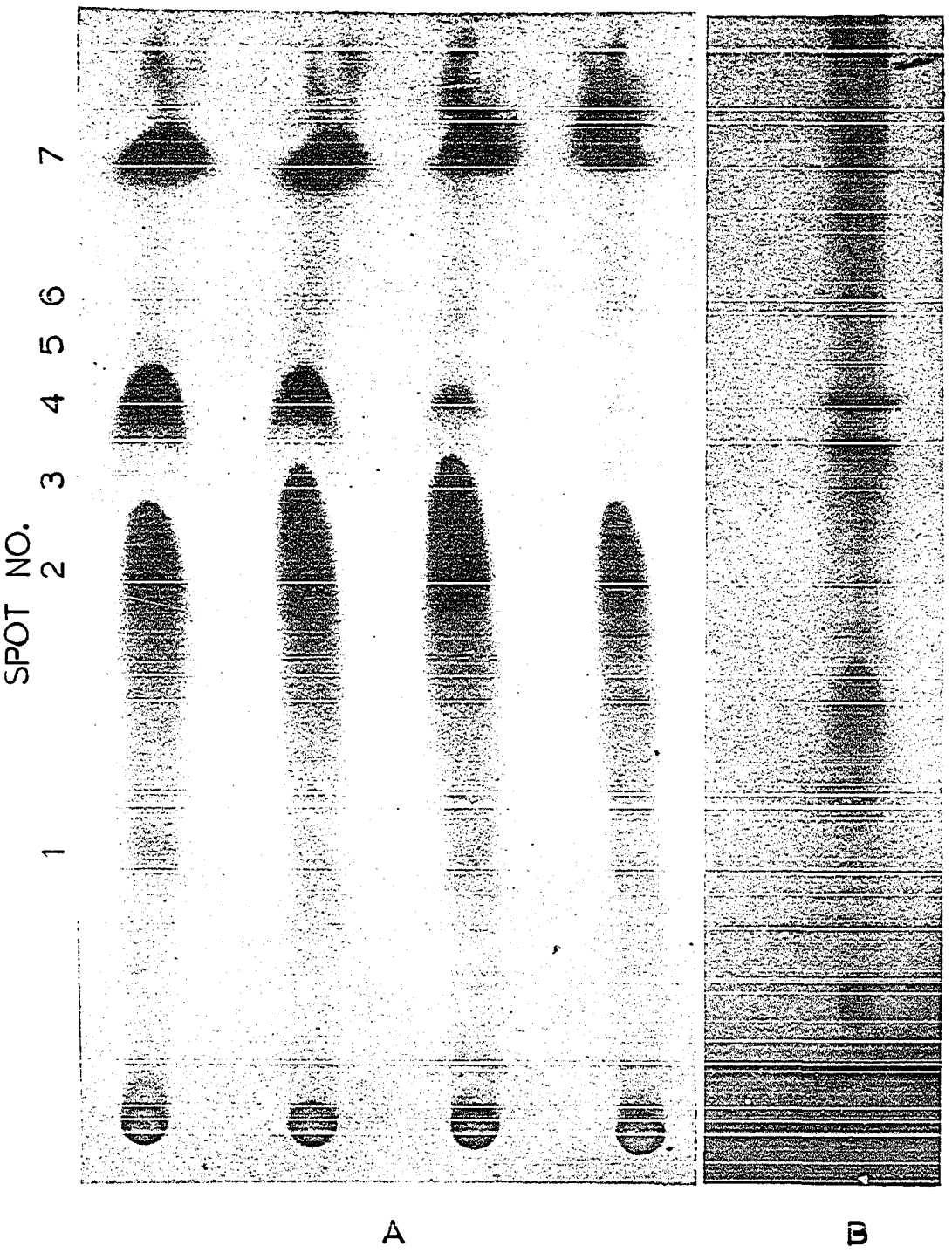

SPOT NO.
1
2
3
4
5
6
7
A
1 2 3 4 5 6 7
B

Figure 4-Aa. Thin-layer chromatograph of Figure 4-A

Spot No. 1. unknown c; 2. unknown b; 3. DIMBOA and HMBOA; 4. unknown a; 5. HBOA; 6. unknown; 7. anthranilic acid-1-^{14}C.

4-Bb. Thin-layer chromatograph of Figure 4-B.

Spot No. 1. unknown b; 2. HMBOA; 3. unknown a; 4. HBOA; 5. 6MBOA; 6. anthranilic acid-1-^{14}C; 7. impurity of anthranilic acid-1-^{14}C.

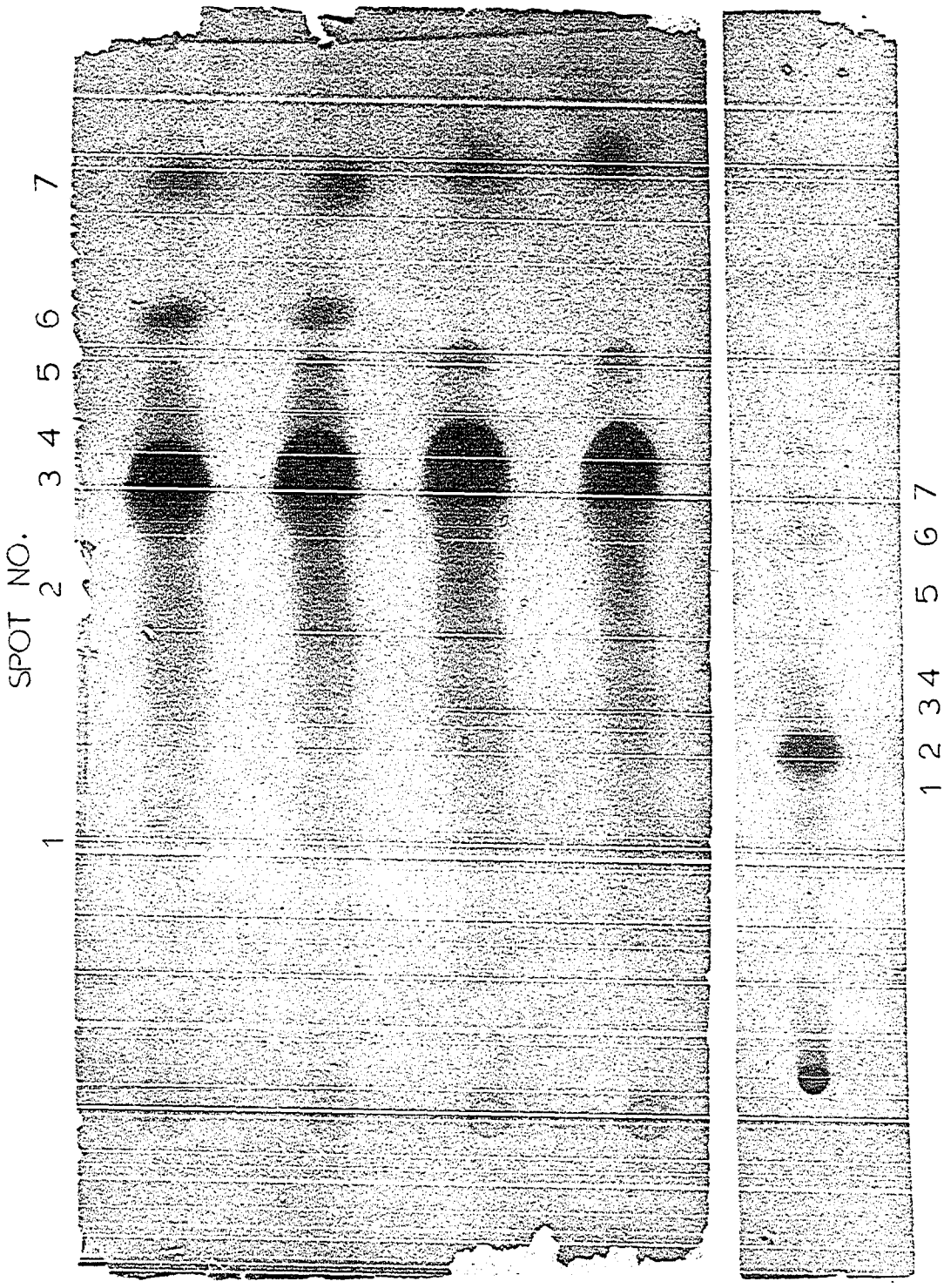

SPOT NO.
1
2
3
4
5
6
7
Aa
1 2 3 4 5 6 7
Bb

Figure 5. Thin-layer chromatograph (Baker-flex silica gel IB-F developed in ether saturated with water) of the products of reaction of anthranilic acid (1 µmole) and E-1.

Reactions conditions: time of reaction: 15 min. I. anthranilic acid and E-1 diluted 1:10 with buffer; II. anthranilic acid and boiled E-; III. anthranilic acid, DIMBOA (1 µmole) and E-1 diluted 1:10 with buffer; IV. anthranilic acid, DIMBOA (1 µmole) and buffer; V. anthranilic acid, HBOA (1 µmole) and E-1 diluted 1:10 with buffer; VI. anthranilic acid, HBOA (1 µmole) and buffer; VII. anthranilic acid, boiled E-1 and E-1 diluted 1:10 with buffer; VIII. E-1 diluted 1:10 with buffer and boiled E-1; Rf values: 0.90, authranilic acid; 0.72, HBOA; 0.68, unknown a; 0.64, DIMBOA (III and IV) or HMBOA (II, VII and VIII).

Rf VALUES

0.64 0.68 0.72 0.90

Figures 3 and 4. They both have the same Rf value relative to DIMBOA (1.1), i.e., Rf of the product/Rf of DIMBOA. Reactions of number V and VI indicate that HBOA does not react with anthranilic acid under the same conditions as DIMBOA.

The nonenzymatic product of anthranilic acid and DIMBOA showed the blue color with the acidic $FeCl_3$ spraying reagent. This nonenzymatic reaction is not affected by ATP, TPNH or PRPP.

Anthranilic acid and cell free extracts of E-2 and E-3

No product has been observed in this experiment, neither enzymatic nor nonenzymatic.

o-Aminophenol-^{14}C and cell free extract E-1

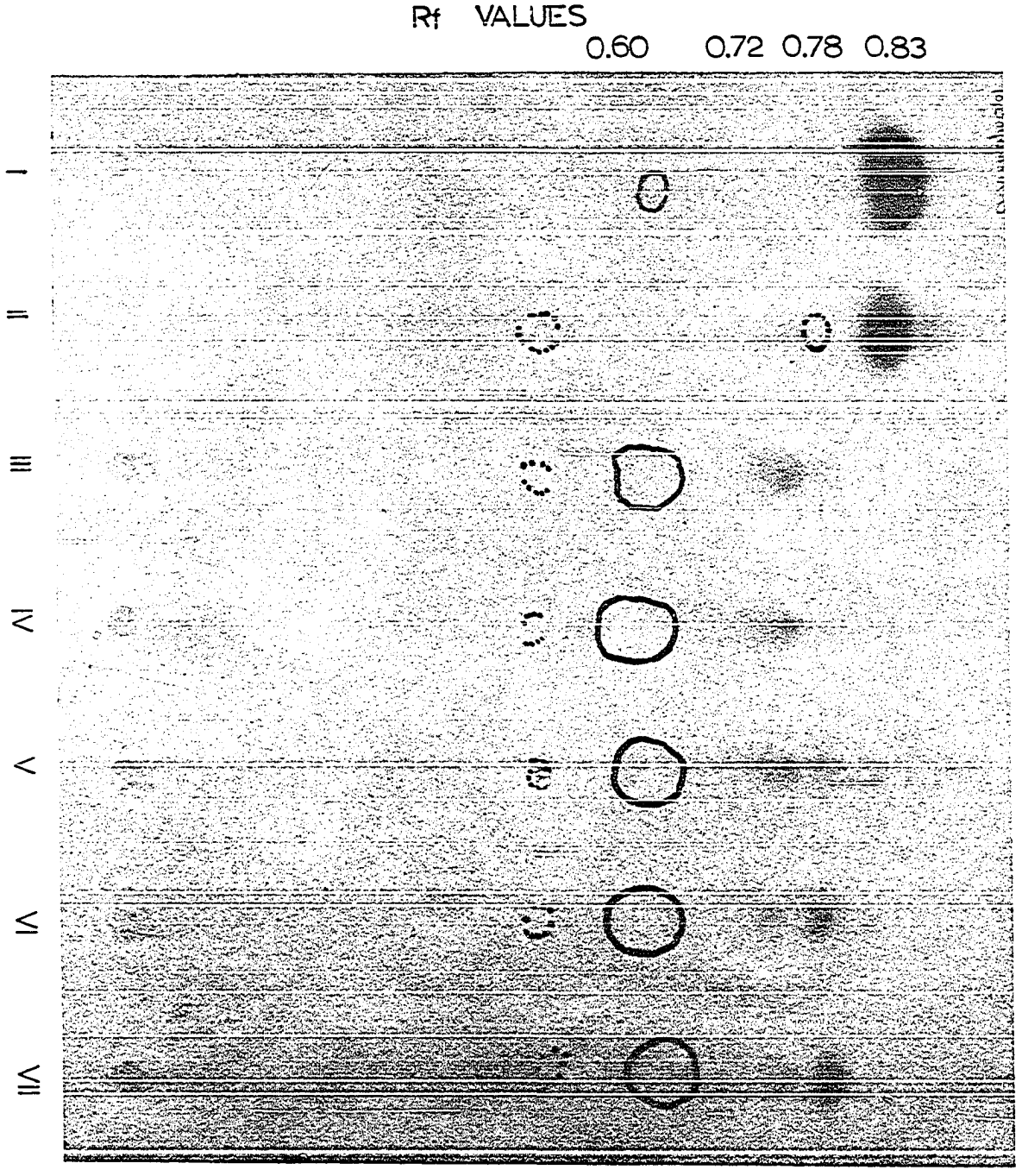
Figure 6 shows the results of incubation of o-aminophenol-^{14}C and the cell-free extract E-1 and shows no ^{14}C in the DIMBOA molecule. Two enzymatic products were observed at Rf 0.78 and Rf 0.72. Neither product has been identified. In the beginning of the reaction, the product at Rf 0.72 was formed. After 40 min, a new product at Rf 0.78 appeared as the concentration of the compound (Rf 0.72) decreased. However, these two products have not been observed in the reaction of o-aminophenol and the cell-free extract E-2 or E-3.

Fixation of Radioactive CO_2 into Corn Seedlings

In these experiments the radioactive DIMBOA was converted to 6MBOA for isolation and determination of specific activity but it should be

Figure 6. Radioautograph of o-aminophenol-^{14}C and cell-free extract E-1

Reaction conditions: o-aminophenol-^{14}C, 1 µmole; ATP, 2 µmoles; PRPP, 2 µmoles; TPN, 2 µmoles; G-6-P, 2 µmole, G-6-P dehydrogenase, 2 units and 1.9 ml of E-1 at 30°C. I. Standard o-aminophenol-^{14}C; II. Control: reaction in boiled E-1; time of reactions: III. 20 min; IV: 40 min; V. 60 min; VI. 80 min; VII. 120 min; Rf values: 0.83, o-aminophenol-^{14}C; 0.78, unknown e; 0.72, unknown d; 0.60, DIMBOA


Rf VALUES
0.60
0.72
0.78
0.83
I
II
III
IV
V
VI
VII

understood that the metabolism being studied is that of DIMBOA. The labeling of HMBOA was also reported. Other compounds of the benzoxazinone family have not been reported due to their extremely low quantities. The duration of the metabolism of radiactive DIMBOA usually took 6 days. During this period, the weight of the corn seedlings generally remained the same and their height varied only within one inch (about 6" to 7").

The time courses of labeling of DIMBOA and HMBOA in four inbred varieties, CI31A, B49, B52 and WF9, are shown in Figures 7, 8, 9, and 10, respectively. Both degradation and synthetic reactions of DIMBOA and HMBOA follow first-order kinetics. The first-order rate constants, k, calculated from least squares for linear function and half-times are listed in Table 2. The average concentration of 6MBOA (or DIMBOA) and

Table 2. Rate constant and half-time for the degradation and synthesis of DIMBOA and HMBOA of four inbred varieties in vivo

	6MBOA		HMBOA	
	Rate constant k (hr^{-1})	Half-time $t_{\frac{1}{2}}$ (hr)	Rate constant k (hr^{-1})	Half-time $t_{\frac{1}{2}}$ (hr)
Degradation				
B52	9.57×10^{-2}	7	2.43×10^{-2}	28
WF9	8.85×10^{-2}	9	3.21×10^{-2}	22
B49	5.07×10^{-2}	14	4.29×10^{-2}	16
CI31A	1.74×10^{-2}	40	1.72×10^{-2}	40
Synthesis				
B52	3.54×10^{-2}	20	3.48×10^{-2}	20
WF9	6.15×10^{-2}	11	3.27×10^{-2}	21
B49	3.17×10^{-2}	22	1.16×10^{-2}	60
CI31A	2.55×10^{-2}	27	2.23×10^{-2}	31

Figure 7. Fixation of radioactive CO_2 into corn seedlings of CI31A

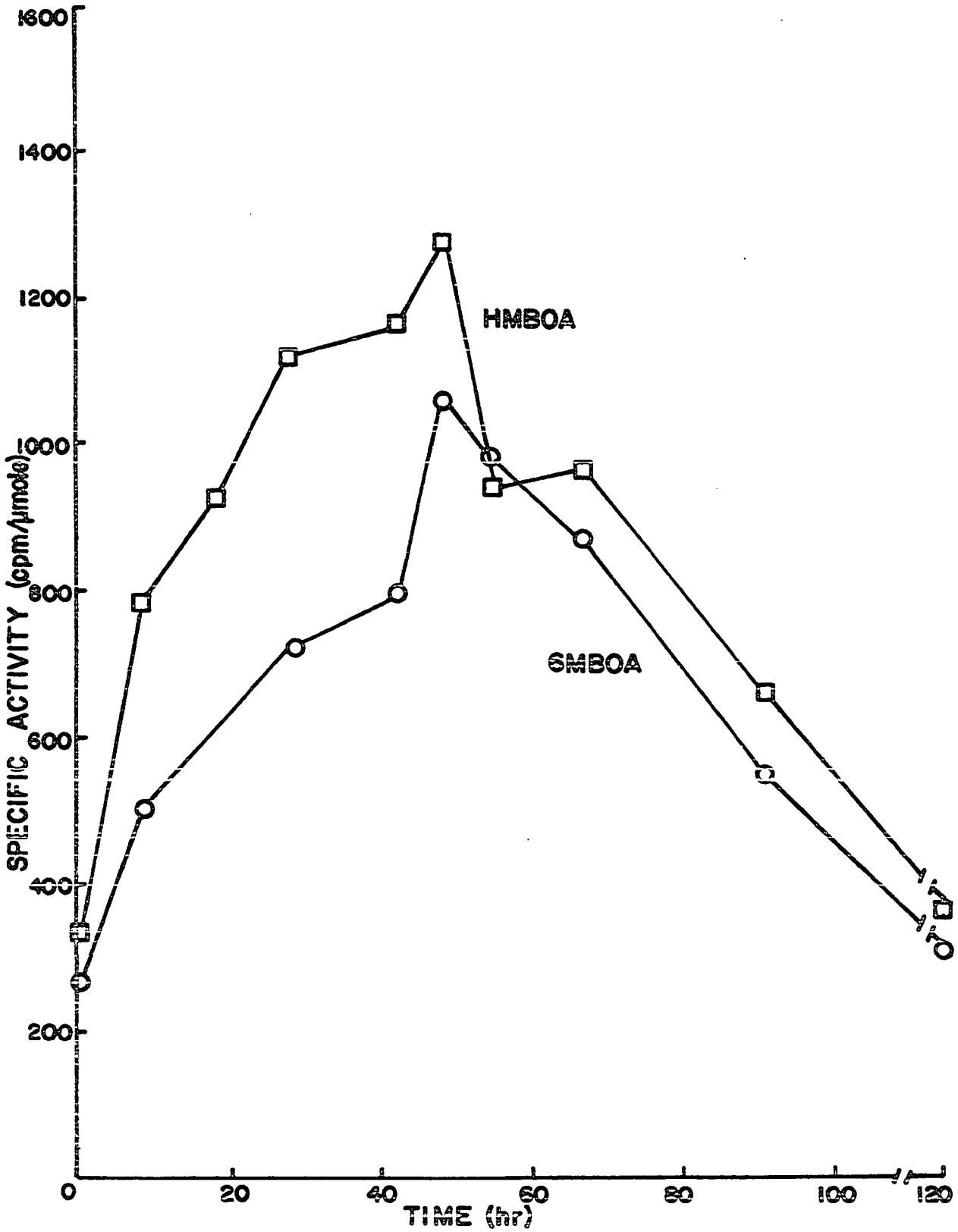

1600
1400
1200
1000
800
600
400
200
0
20
40
60
80
100
120
SPECIFIC ACTIVITY (cpm/μmole)
TIME (hr)
HMBOA
6MBOA

Figure 8. Fixation of radioactive CO_2 into corn seedlings of B49

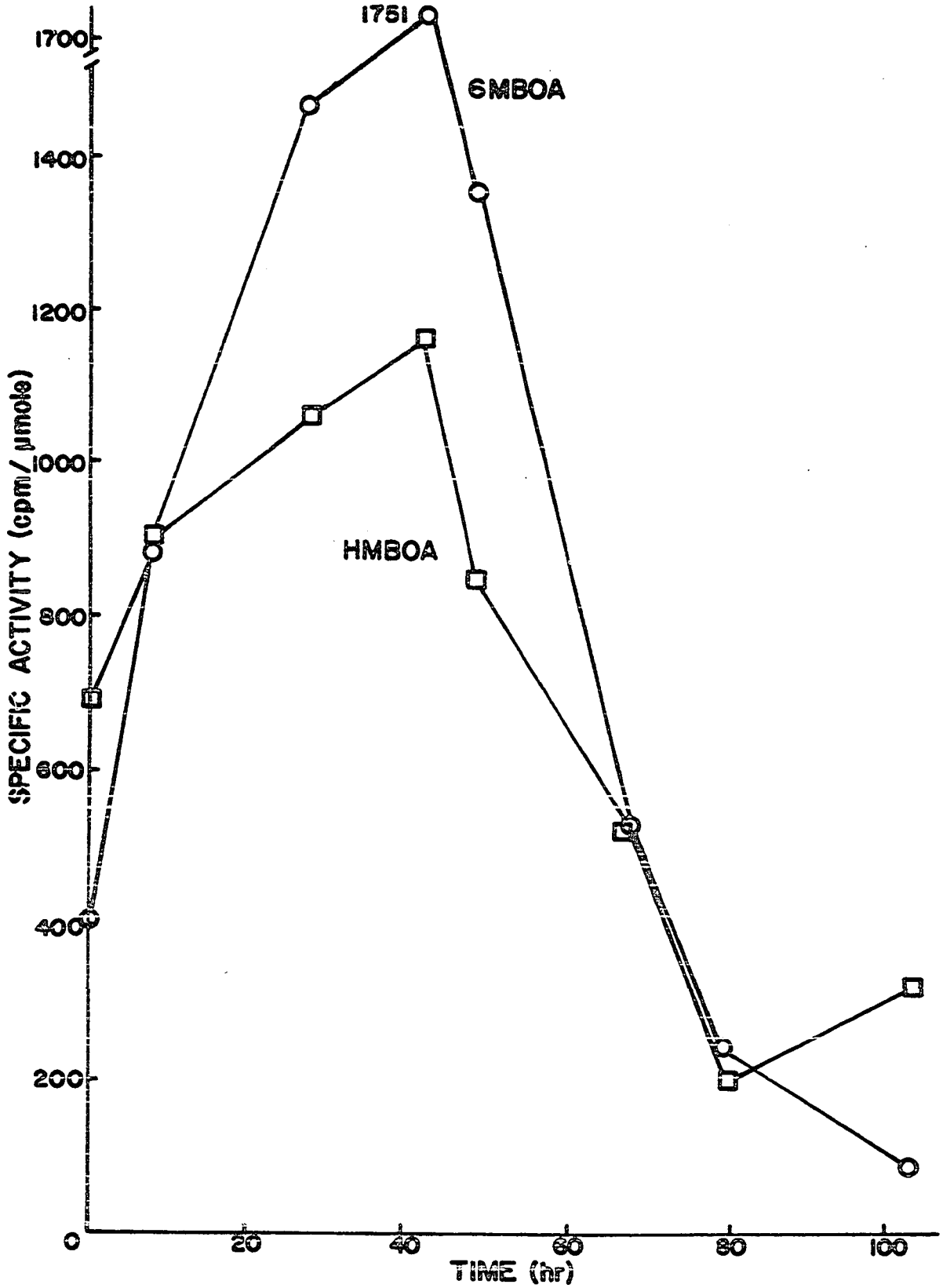

1751
6MBOA
HMBOA
SPECIFIC ACTIVITY (cpm/μmole)
TIME (hr)
0
200
400
600
800
1000
1200
1400
1700
20
40
60
80
100

Figure 9. Fixation of radioactive CO_2 into corn seedlings of B52

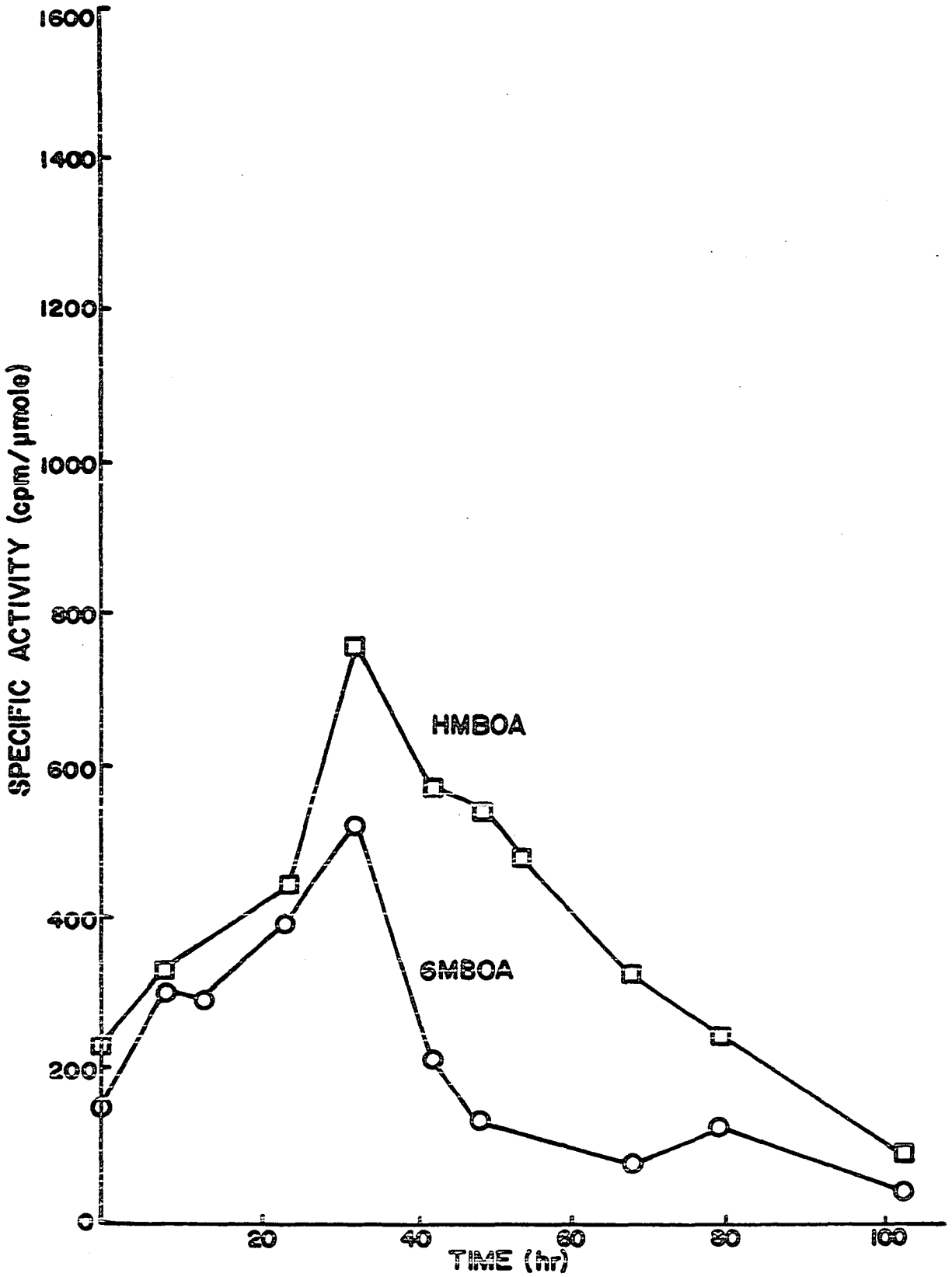
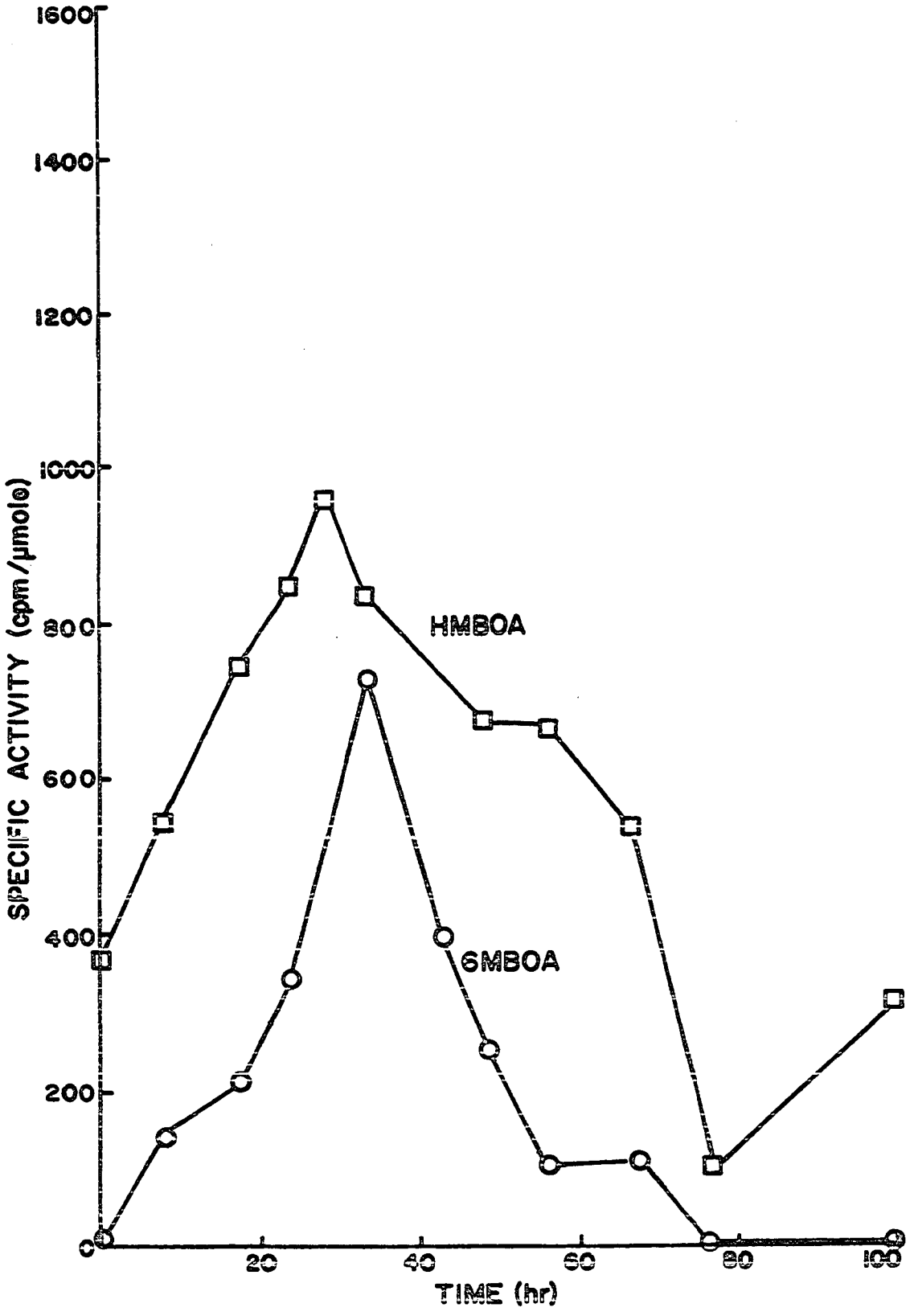

SPECIFIC ACTIVITY (cpm/μmole)
1600
1400
1200
1000
800
600
400
200
0
HMBOA
6MBOA
20
40
60
80
100
TIME (hr)

Figure 10. Fixation of radioactive CO_2 into corn seedlings of WF9


1600
1400
1200
1000
800
600
400
200
0
SPECIFIC ACTIVITY (cpm/μmole)
HMBOA
6MBOA
20
40
60
80
100
TIME (hr)

of HMBOA in young corn seedlings (about 6") are listed in Table 3. The content of 6MBOA in the plant remained at nearly constant during the period of experiment. B49 and CI31A have higher maximum specific activities of 6MBOA and HMBOA than that of B52 and WF9 as shown in Table 3.

Table 3. The average concentrations of 6MBOA (DIMBOA) and HMBOA in corn seedlings of four inbred varieties and their maximum specific activities

	6MBOA		HMBOA	
	umole/g seedling	Maximum Specific activity cpm/umole	umole/g seedling	Maximum Specific activity cpm/umole
B49	1.28	1,751	0.41	1,172
B52	0.81	531	0.40	762
CI31A	0.56	1,053	0.39	1,282
WF9	0.22	733	0.22	974

Enzymatic Reduction of HBOA and the Activity of β-Glucosidase

Activity of 2-hydroxy-benzoxazinone reductase

The activity of 2-hydroxy-benzoxazinone reductase in CI31A, B49, and WF9 are shown in Figure 11, 12, and 13, respectively, and in Table 4. Figure 11 shows that the activity of the enzyme is stimulated by TPNH, not TPN. It was shown previously (17) that the activity of the enzyme was higher in the presence of TPNH than without any cofactors. The enzyme from WF9 shows a long lag period after which the reaction rate is similar to that of the enzymes from CI31A and B49.

Figure 11. Activity of 2-hydroxy-benzoxazinone reductase in CI31A at 37°C in phosphate buffer (pH 7.0)

A. HBOA, 1 umole; TPN, 2 umoles; G-6-P, 2 umoles; G-6-P dehydrogenase, 2 units; and E-1, 2 ml; total volume 2.5 ml

B. HBOA, 1 umole; TPN, 2 umole; and E-1, 2 ml; total volume 2.5 ml

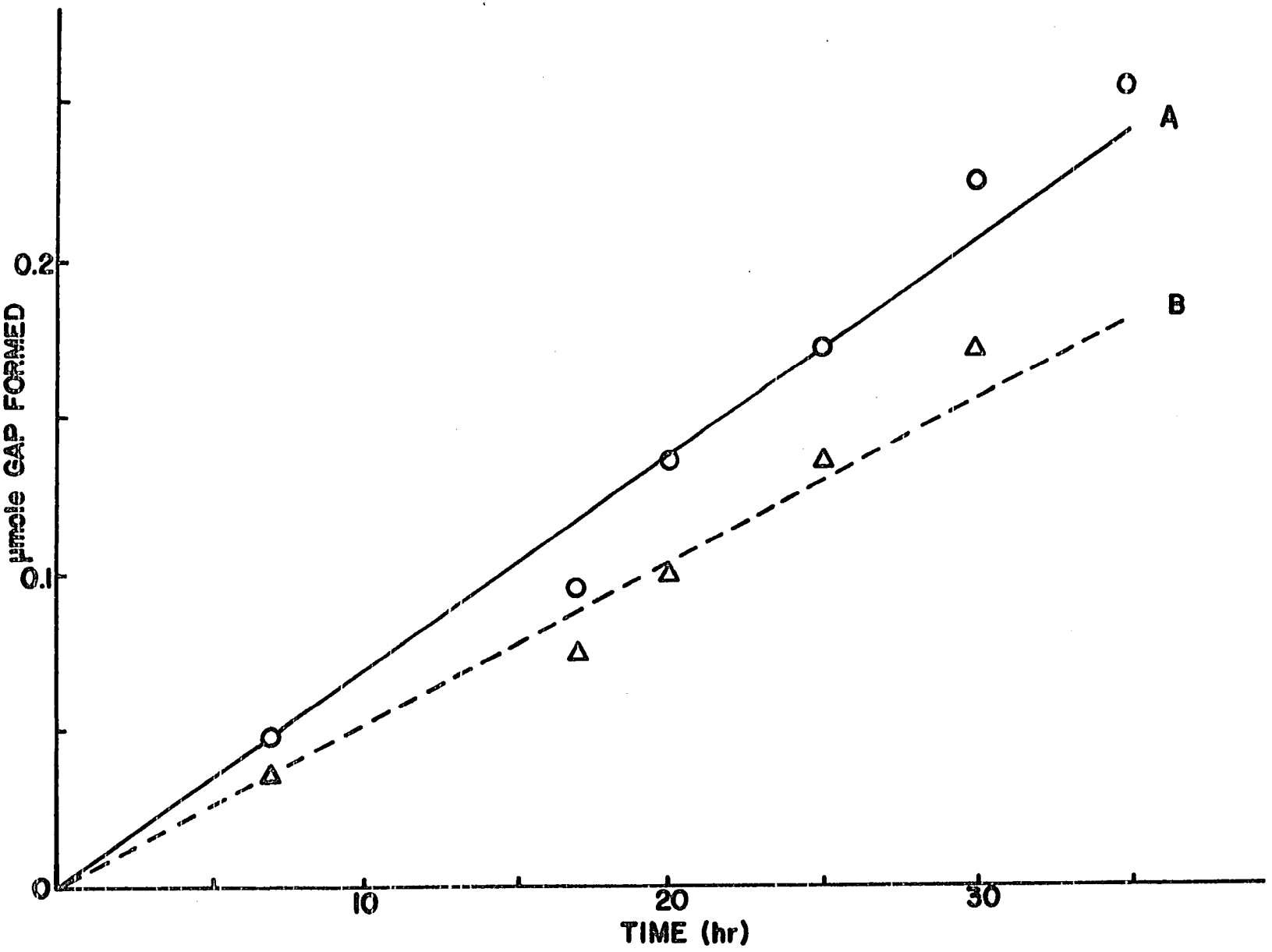

μmole GAP FORMED
0
0.1
0.2
10
20
30
TIME (hr)
A
B

Figure 12. Activity of 2-hydroxy-benzoxazinone reductase in B49 at 37°C in phosphate buffer (pH 7.0)

HBOA, 1 umole; TPN, 2 umoles; G-6-P, 2 umoles; G-6-P dehydrogenase, 2 units; and E-1, 2 ml; total volume 2.5 ml

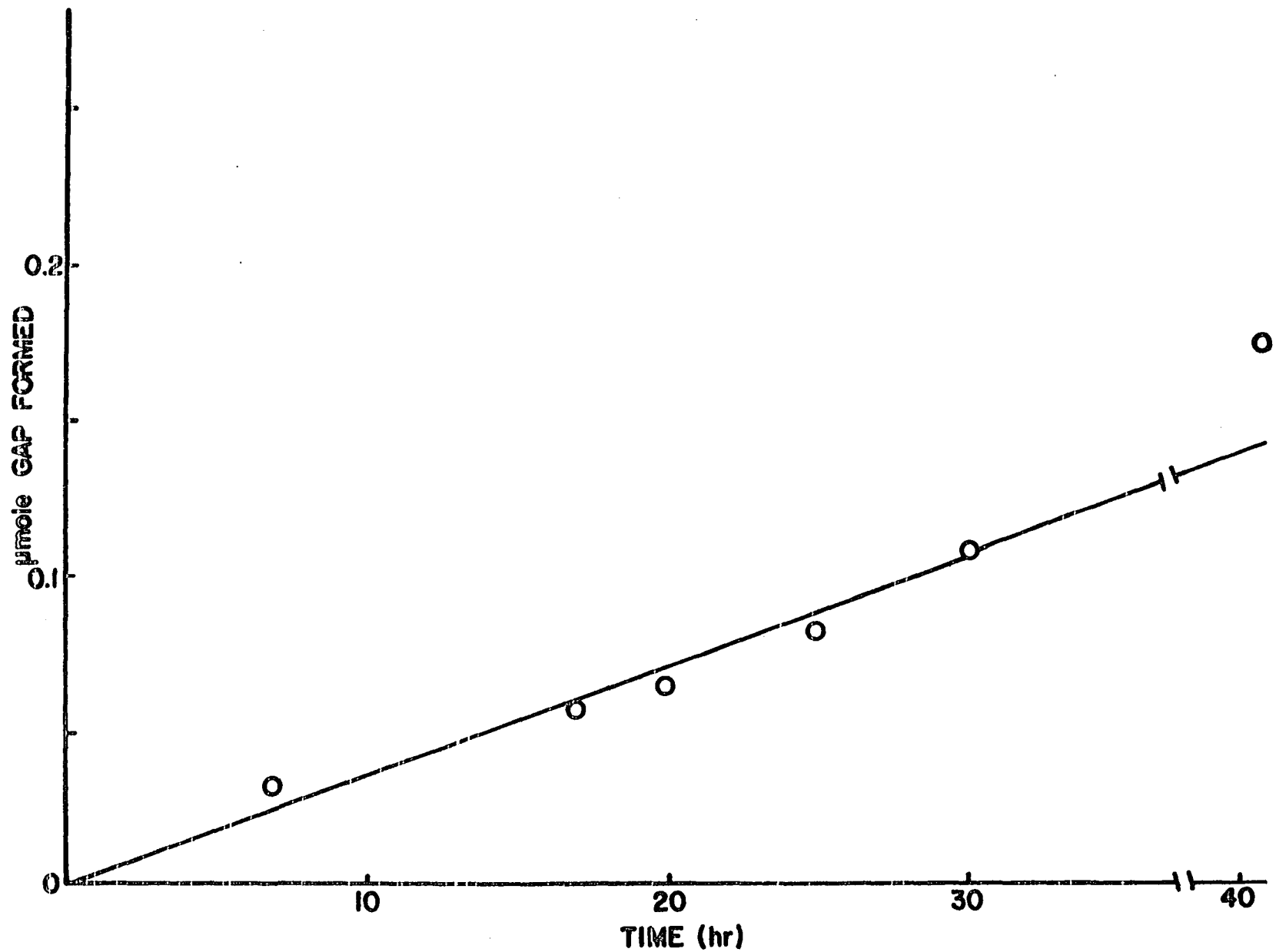

0.2
0.1
0
μmole GAP FORMED
10
20
30
40
TIME (hr)

Figure 13. Activity of 2-hydroxy-benzoxazinone reductase in WF9 at 37°C in phosphate buffer (pH 7.0)

HBOA, 1 umole; TPN, 2 umoles; G-6-P, 2 umoles; G-6-P dehydrogenase, 2 units; and E-1, 2 ml; total volume 2.5 ml

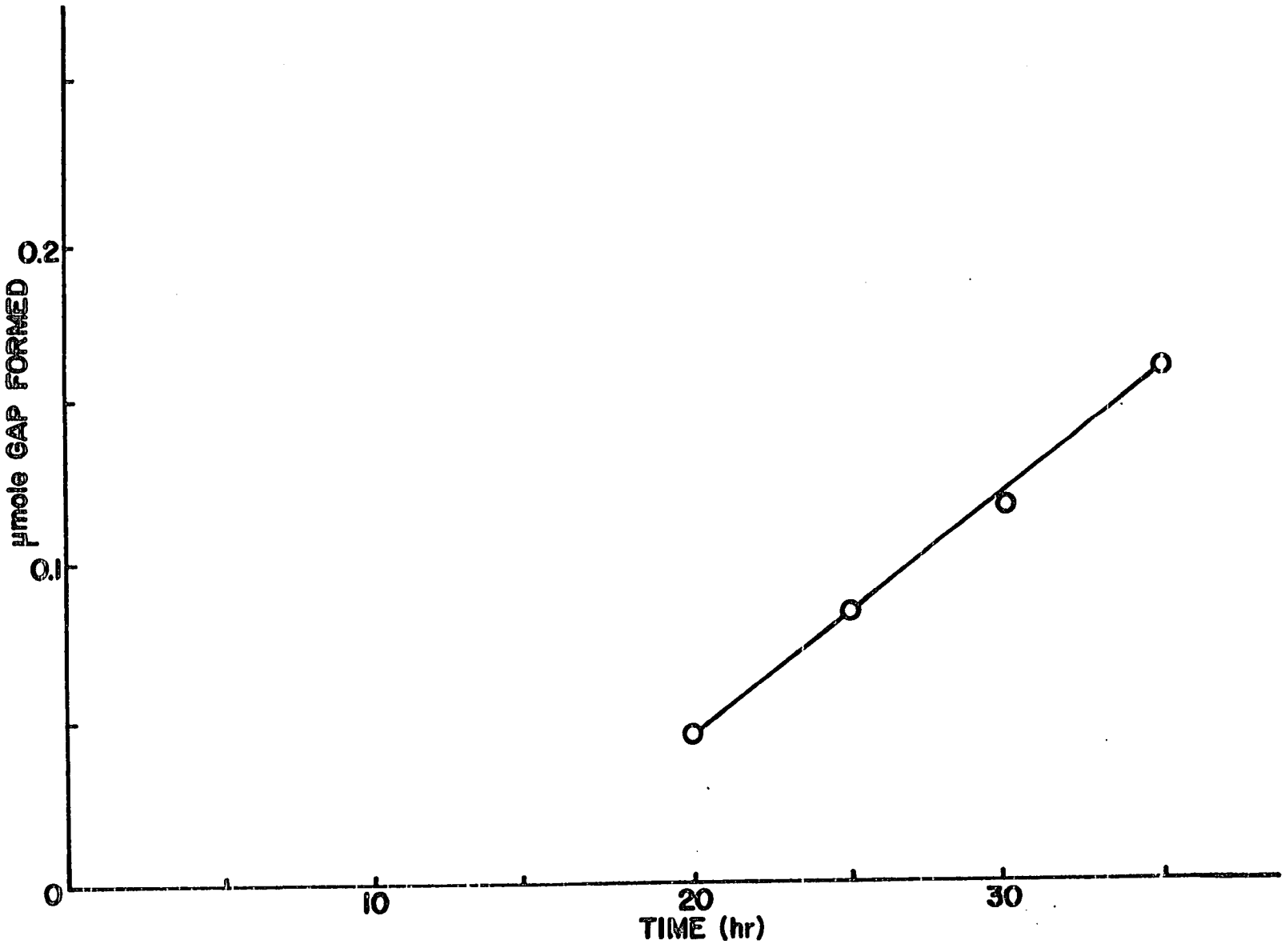

0.2
0.1
0
μmole GAP FORMED
10
20
30
TIME (hr)

Table 4. Specific activity of 2-hydroxy-benzoxazinone reductase

	mg protein/ g seedling	mg protein/ ml extract	μmole/hr	specific activity μmole/hr/mg protein
CI31A	2.9	1.5	6.80×10^{-3}	4.54×10^{-3}
B49	2.7	1.4	3.63×10^{-3}	2.60×10^{-3}
WF9	4.0	2.0	7.80×10^{-3}	9.90×10^{-3}

β-glucosidase

All three inbred varieties of CI31A, B49, and WF9 have the same specific activities of β-glucosidase as shown in Table 5.

Table 5. Specific activity of β-glucosidase

	mg protein g seedling	mg protein/ ml extract	O.D. (410 nm)/min	umole o-nitro-phenol/min/ml	Specific activity μmole/min/mg protein
CI31A	2.9	1.5	0.041	0.70	0.47
B49	2.7	1.4	0.038	0.67	0.48
WF9	4.0	2.0	0.046	0.80	0.40

Chemical Aspects of Benzoxazinones

Detoxification of Simazine by DIMBOA *in vitro*

The study of the time-course of the detoxification of simazine by DIMBOA at two concentrations differing by a factor of 10 is shown in the radioautograph of Figure 14 and Table 6. The results show that the rate

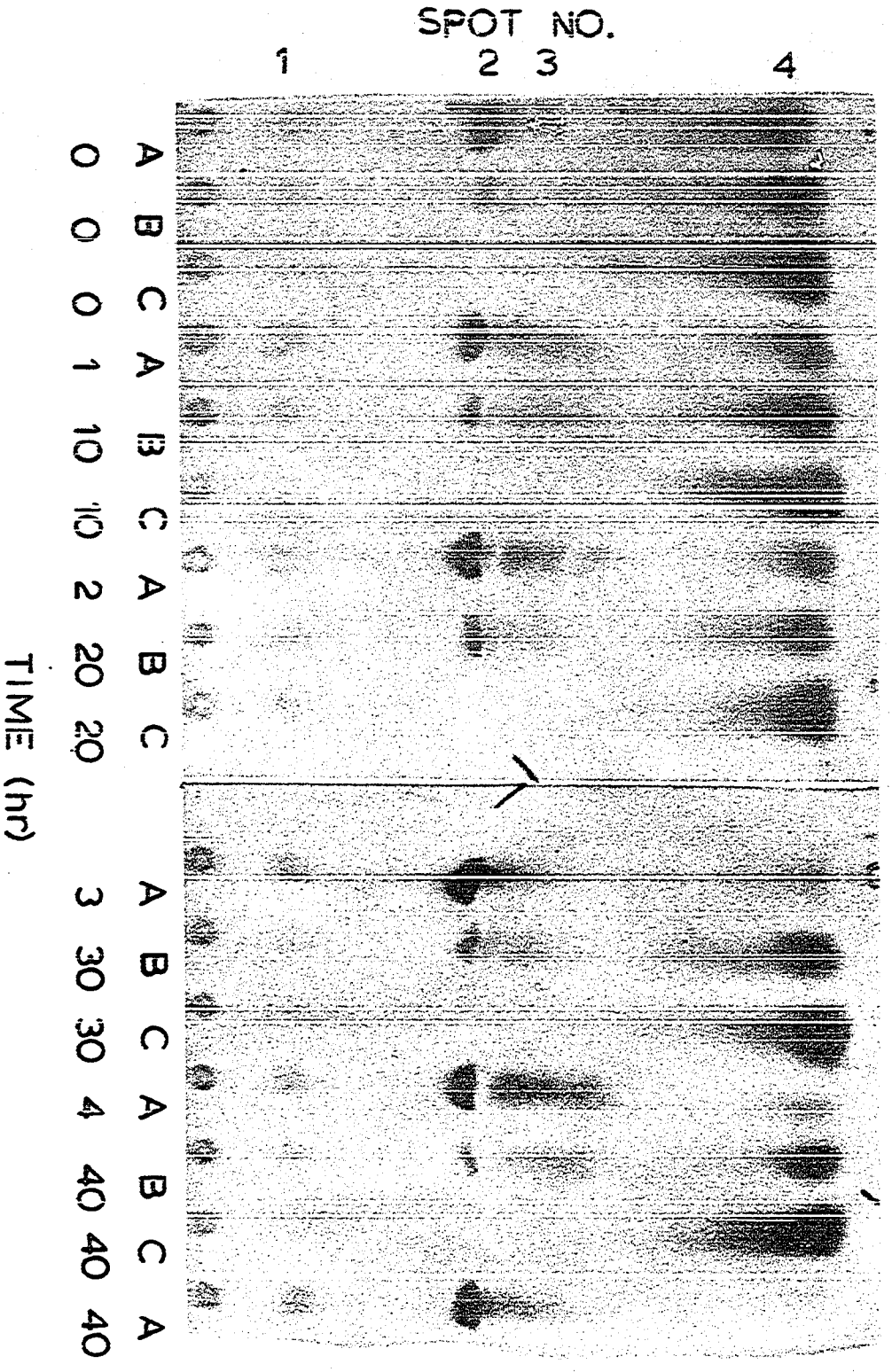
Figure 14. Radioautograph of the detoxification of simazine catalyzed by DIMBOA at 37°C

A. DIMBOA, 4 μmoles, and simazine, 20 mμmoles, in 2 ml citrate buffer (pH 4.8)

B. DIMBOA, 0.4 μmoles, and simazine, 20 mμmoles, in 2 ml citrate buffer (pH 4.8)

C. Simazine, 20 mμmoles, in 2 ml citrate buffer (pH 4.8)

Spot No. 1. unknown; 2. hydroxysimazine; 3. unknown; 4. simazine


SPOT NO.
1
2 3
4
A B C A B C A B C A B C A B C A
0 0 0 1 10 10 2 20 20 3 30 30 4 40 40 40
TIME (hr)

of detoxification of simazine is dependent on the concentration of DIMBOA. The hydrolysis of simazine started rapidly at the higher concentration of DIMBOA. At the high concentration of DIMBOA (2.0 mM), the hydrolysis was complete after 40 hours. At the low concentration of DIMBOA (0.2 mM), 70% of simazine was not hydrolyzed even after 40 hours.

Table 6. Effect of concentration of DIMBOA on rate of hydrolysis of simazine

Conc. DIMBOA	Simazine recovered, % of total radioactivity				
	t x C, mmoles/l x hr[a]				
	0[b]	2	4	6	8
0	91	91	93	93	91
0.2 mM	82	68	65	72	64
2.0 mM	53	45	32	14	15

[a]Product of concentration of DIMBOA and time of incubation.

[b]Samples taken as soon as possible after preparation of reaction mixtures.

Degradation of DIMBOA in Hydrochloric Acid Solution

The change of the UV absorbance spectrum of DIMBOA in 1 N HCl solution at 37°C is shown in Figure 15. The reaction was complete after 5 days. The final products were purified by silica gel GF_{254} TLC and they were isolated from Rfs 0.95, 0.90 and 0.20. Their UV absorbance spectra are shown in Figure 16 for Rf 0.95, Figure 17 for Rf 0.90, and Figure 18 for Rf 0.20. When these three compounds were mixed together, a UV spectrum similar to the spectrum at 5 days in Figure 15 was obtained.

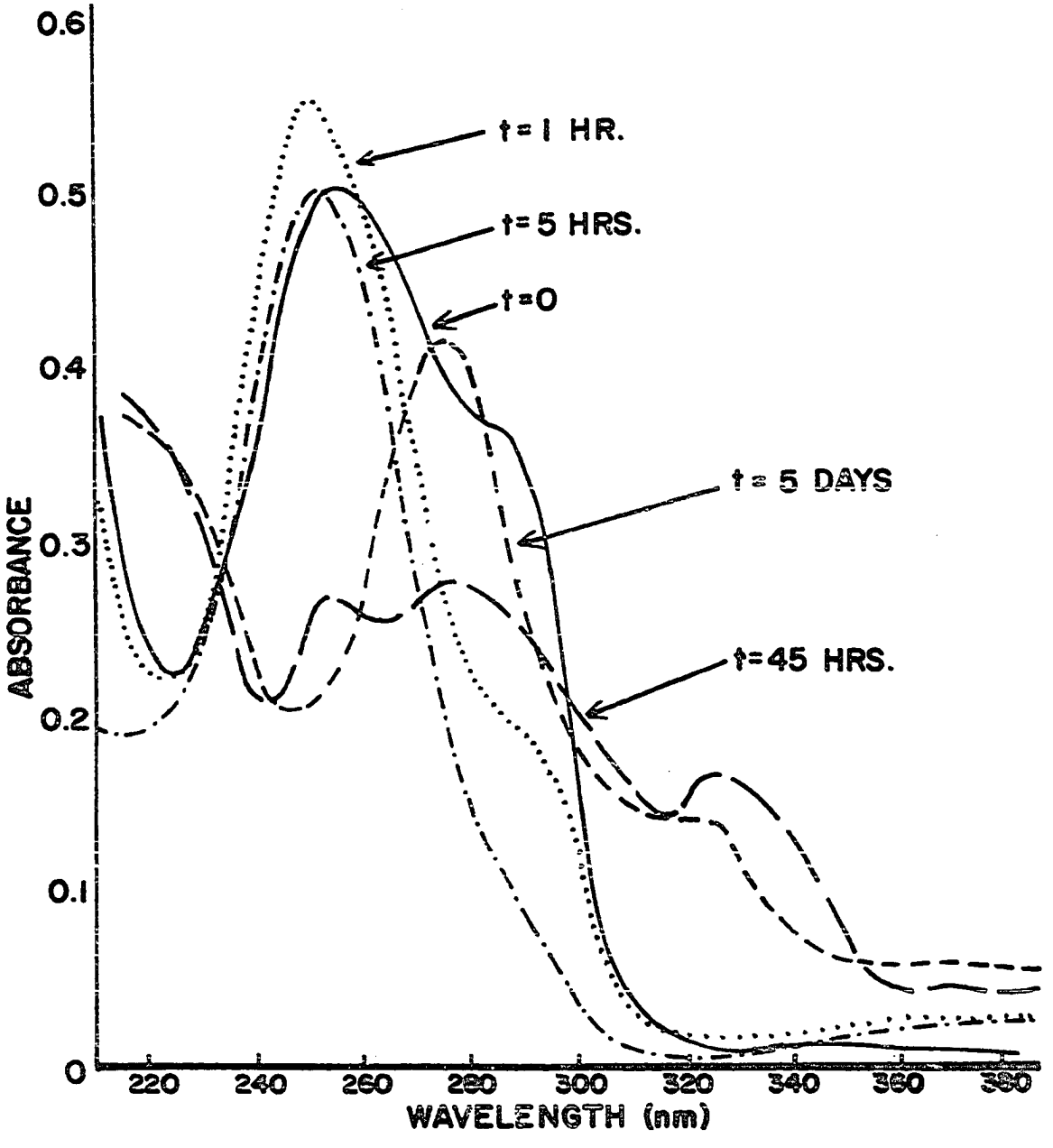


Figure 15. Degradation of DIMBOA in 1 N HCl (0.01 mg/ml) at 37°C

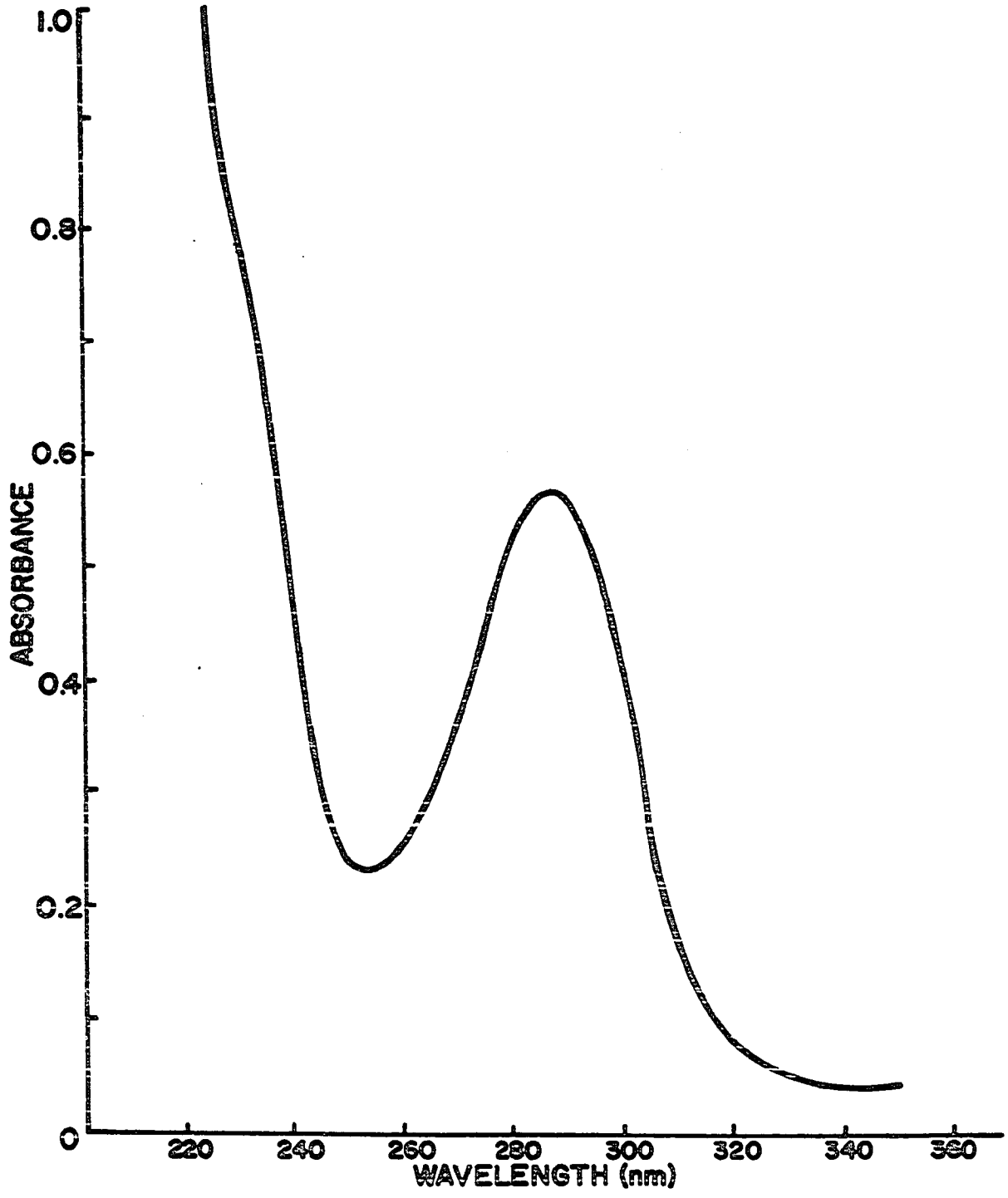


Figure 16. UV spectrum of the acid-catalyzed DIMBOA degradation product at Rf = 0.95 in 1 N HCl

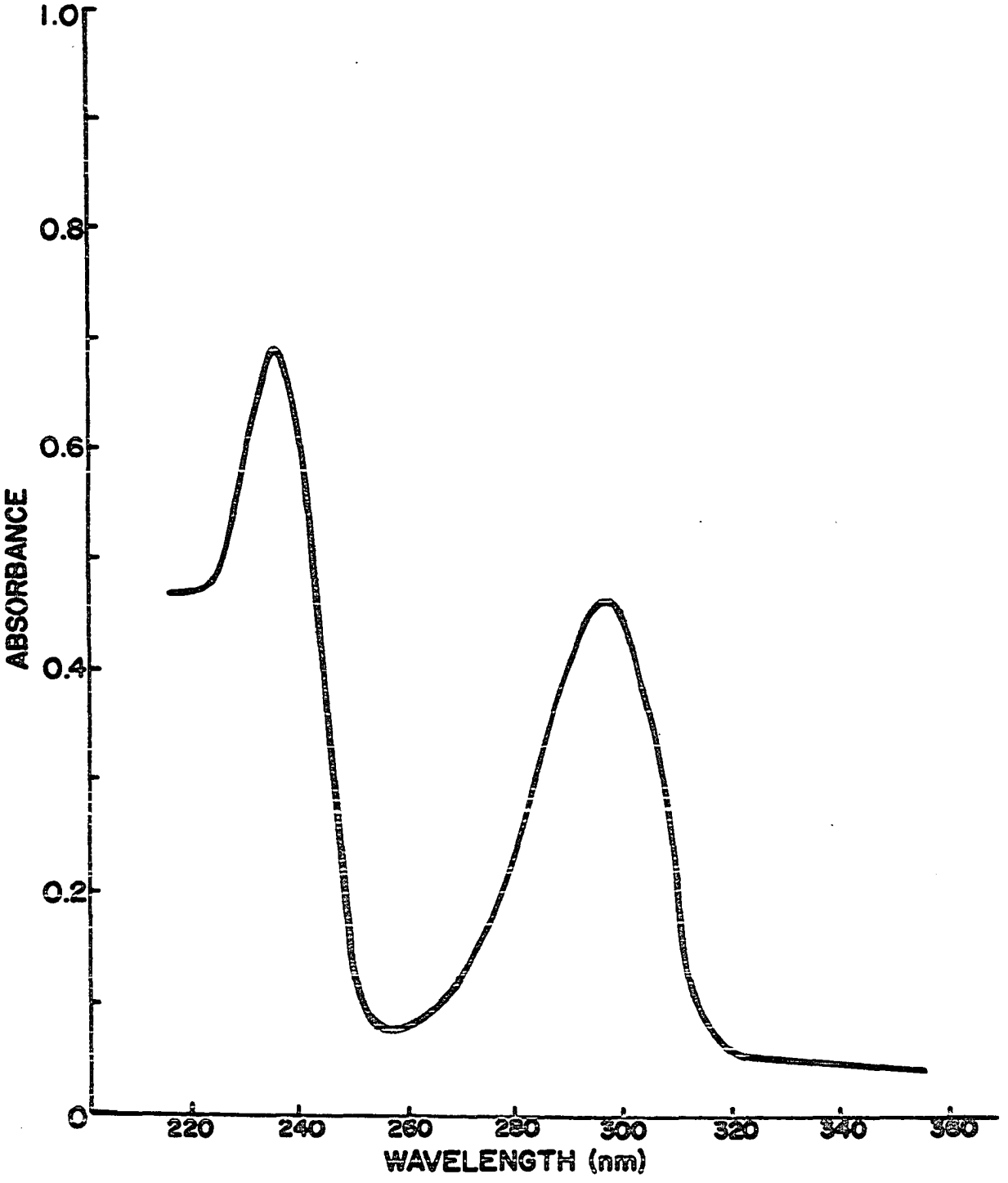


Figure 17. UV spectrum of the acid-catalyzed DIMBOA degradation product at Rf = 0.90 in 1 N HCl

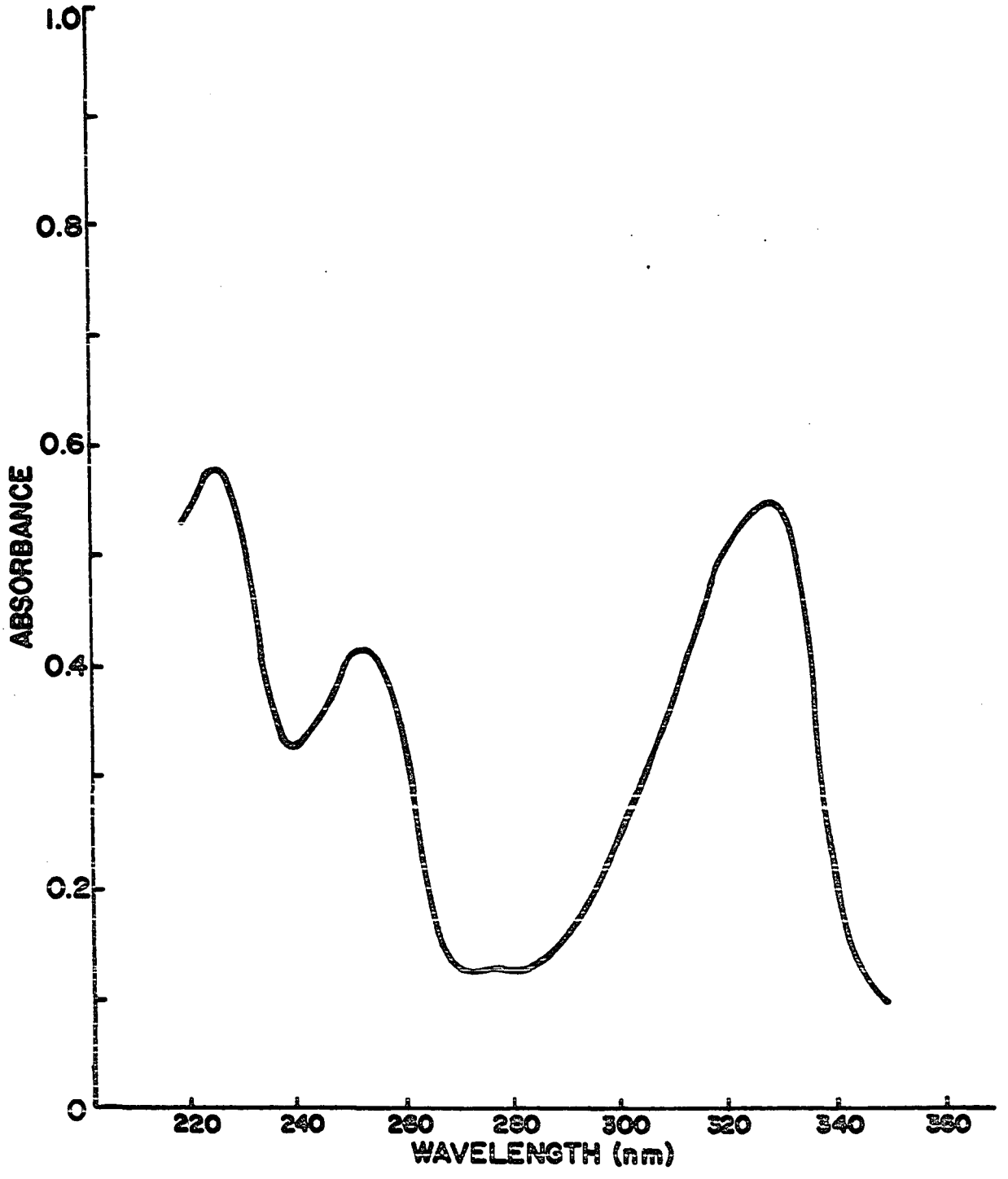


Figure 18. UV spectrum of the acid-catalyzed DIMBOA degradation product at Rf = 0.20 in 1 N HCl

The product at Rf 0.90 has the same UV spectrum as 6MBOA (Appendix Figure A-11). Its structure was also confirmed as 6MBOA by the mass spectrum.

In the mass spectrum of the product at Rf 0.20 the parent peak has m/e = 255, and a p + 2 peak with about one-third the intensity of the parent peak indicates the presence of Cl in the molecule. According to the report by Coutts and Pound (69), when hydrochloric acid and other nucleophilic reagents react with aromatic hydroxylamines, para-chlorine substituted amines are the major products. Since the position para to the DIMBOA hydroxamate has been occupied by the methoxy group, therefore, ortho-substitution is expected. The complete structure of this product is not known.

The structure of the product at Rf 0.95 (Figure 16) has not beben confirmed. No reliable mass spectrum could be obtained for this product. Since it has a UV spectrum similar to that of 5-methoxy-o-aminophenol (Appendix Figure A-12), it is suggested that this product could be 5-methoxy-o-aminophenol or a derivative.

6MBOA was stable in 1 N HCl solution at 60°C for 2 days whereas HBOA was completely converted to o-aminophenol which was identified by the UV spectrum.

Chemical Synthesis of DIBOA

The overall reaction scheme for the synthesis of DIBOA is summarized in Figure 19.

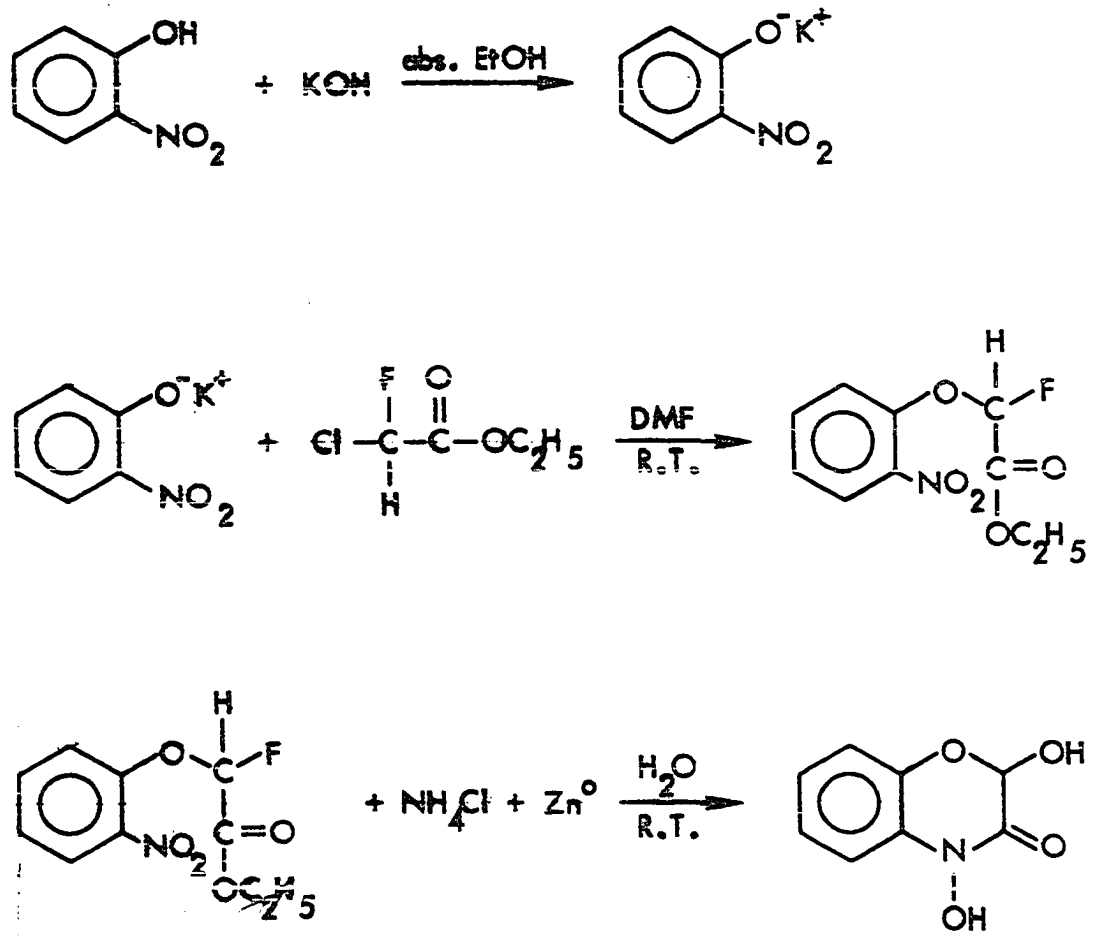


Figure 19. Synthesis of DIBOA

The UV spectrum of ethyl o-nitrophenoxyfluoroacetate is shown in Appendix Figure A-6 and its extinction coefficient is listed in Appendix Table A-1. The mass spectrum shows a molecular ion at m/e 243 (calculated molecular weight 243.2), as shown in Figure 20, and fragment ions consistent with the proposed structure. This compound is very stable in

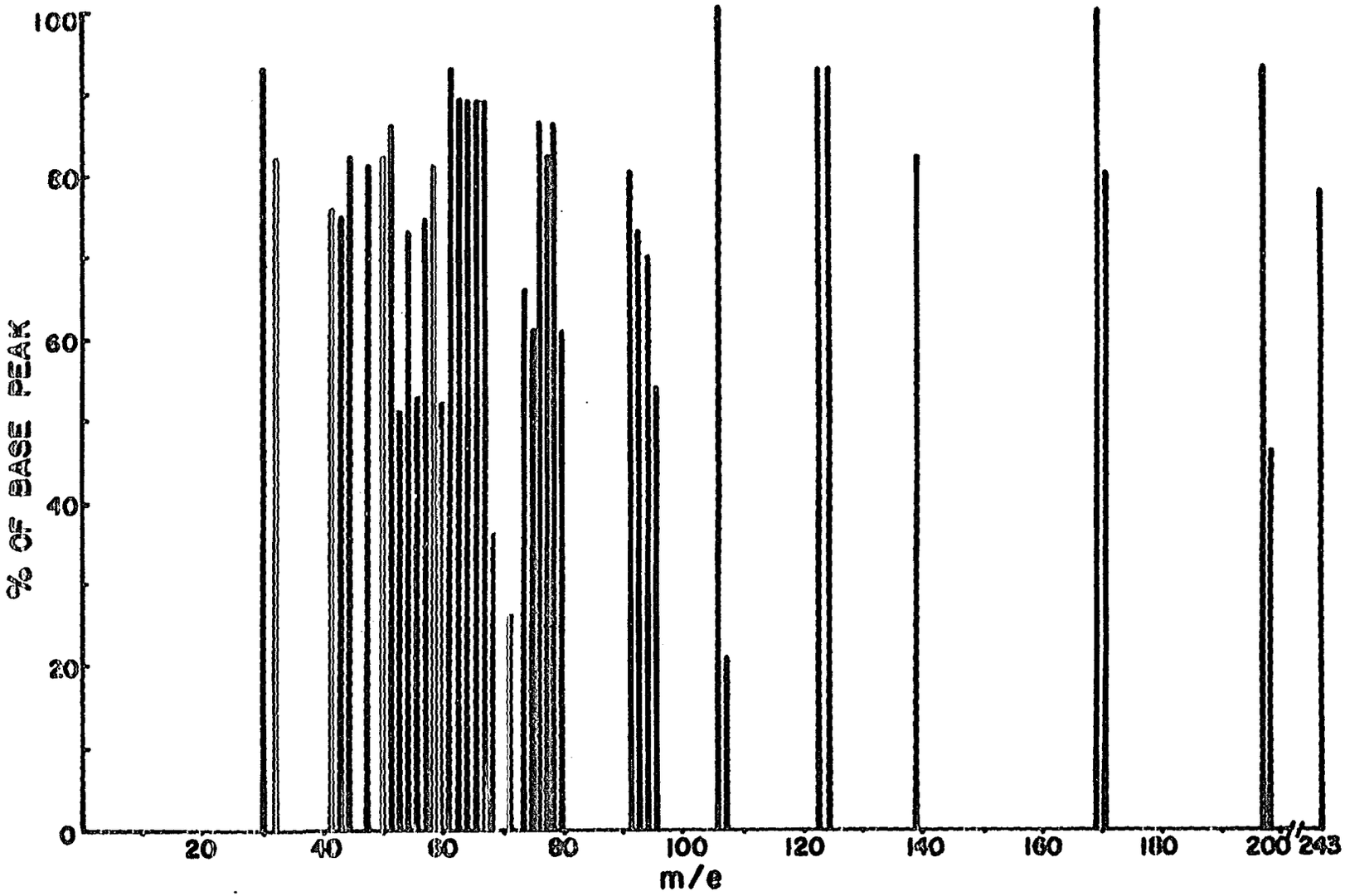


Figure 20. Mass spectrum of o-nitrophenoxyfluoroacetate

water or NH_4Cl solution at room temperature, but it decomposes at temperatures above 100°C and the melting point was unable to be detected. Its density is 1.2963 g/ml.

The UV spectrum of DIBOA is shown in Appendix Figure A-4 and its extinction coefficient is listed in Appendix Table A-1. The mass spectrum is shown in Figure 21. A molecular ion at m/e 181 and fragment ions consistent with the proposed structure are seen. The molecular weight by high resolution mass spectroscopy is 181.037325 (calculated molecular weight 181.0375024 based on the standard ^{12}C = 12.00000). BOA was observed when the synthetic DIBOA was degraded in water. DIBOA and DIMBOA are unstable on silicic acid. The instability is demonstrated by the change in their UV spectra as shown in Figures 22 and 23.

Miscellaneous Reactions

The reactions for the synthesis of 2-o-aminophenoxy-4-hydroxy-1,4(2H)-benzoxazin-3-one(AHBOA) are shown in Figure 25. The NMR spectrum of methyl bis-(o-nitrophenoxy)-acetate has a singlet at 3.8 δ due to the methyl group and a singlet at 6.3δ from the proton at the acetal carbon atom. The aromatic protons have a complex split pattern between 7.0 and 8.0δ. The mass spectrum of this compound has the maximum mass m/e 210 and no peaks at higher mass.

The mass spectrum of AHBOA has a molecular ion at m/e 272 (calculated molecular weight 272.11) from which the structure of the compound was proposed. The UV spectrum has maxima at 283 nm and 237 nm in 95% ethanol with both peaks skewed, but a spectrum similar to that of DIBOA, with more symmetrical peaks, was obtained in acid solution as shown in Figure 24.

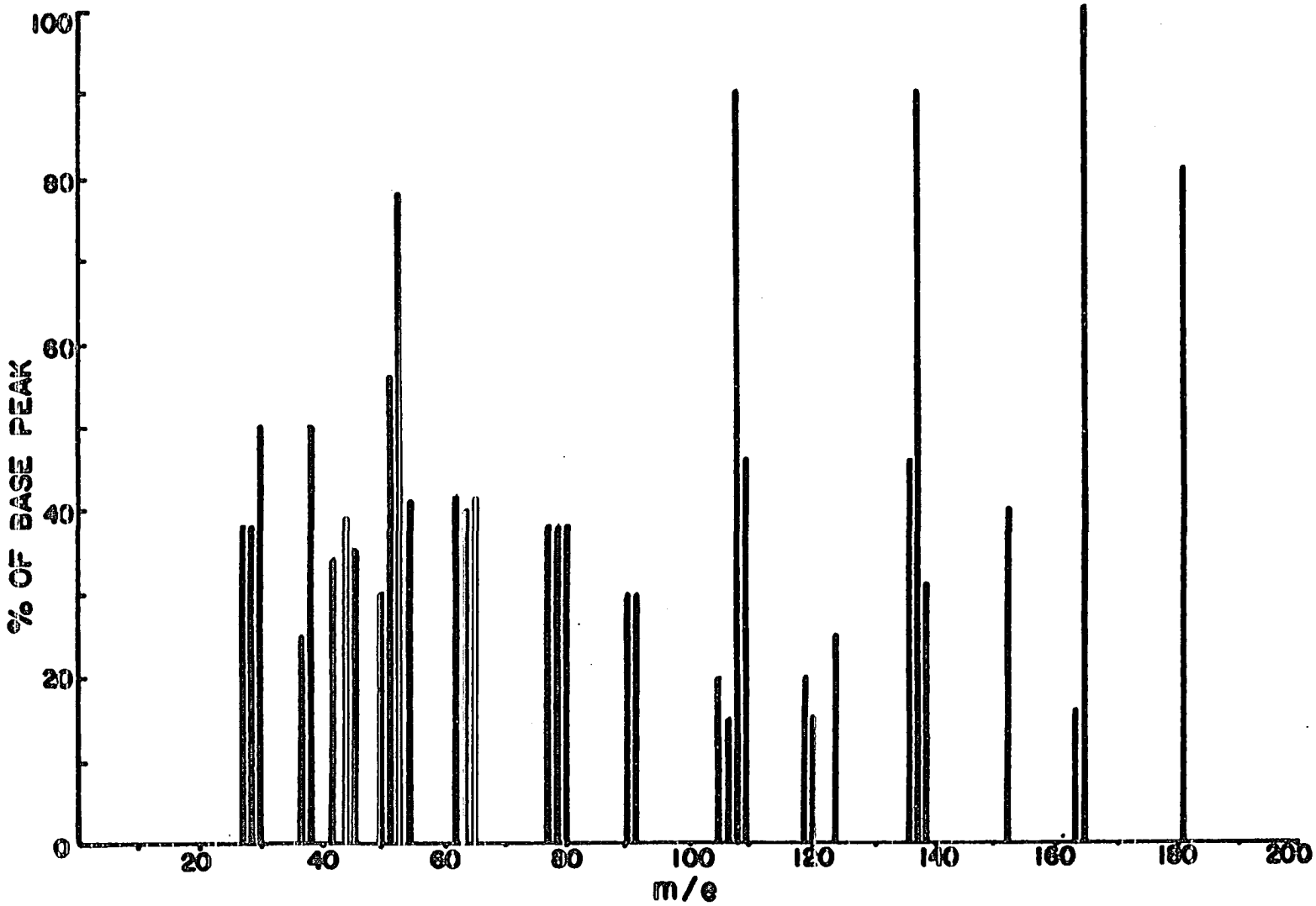


Figure 21. Mass spectrum of DIBOA

Figure 22. The change of UV spectrum of DIBOA on silica gel TLC

I. DIBOA

II. DIBOA (same concentration as I) on TLC for 2 hours

III. DIBOA (same concentration as I) on TLC for 6 hours

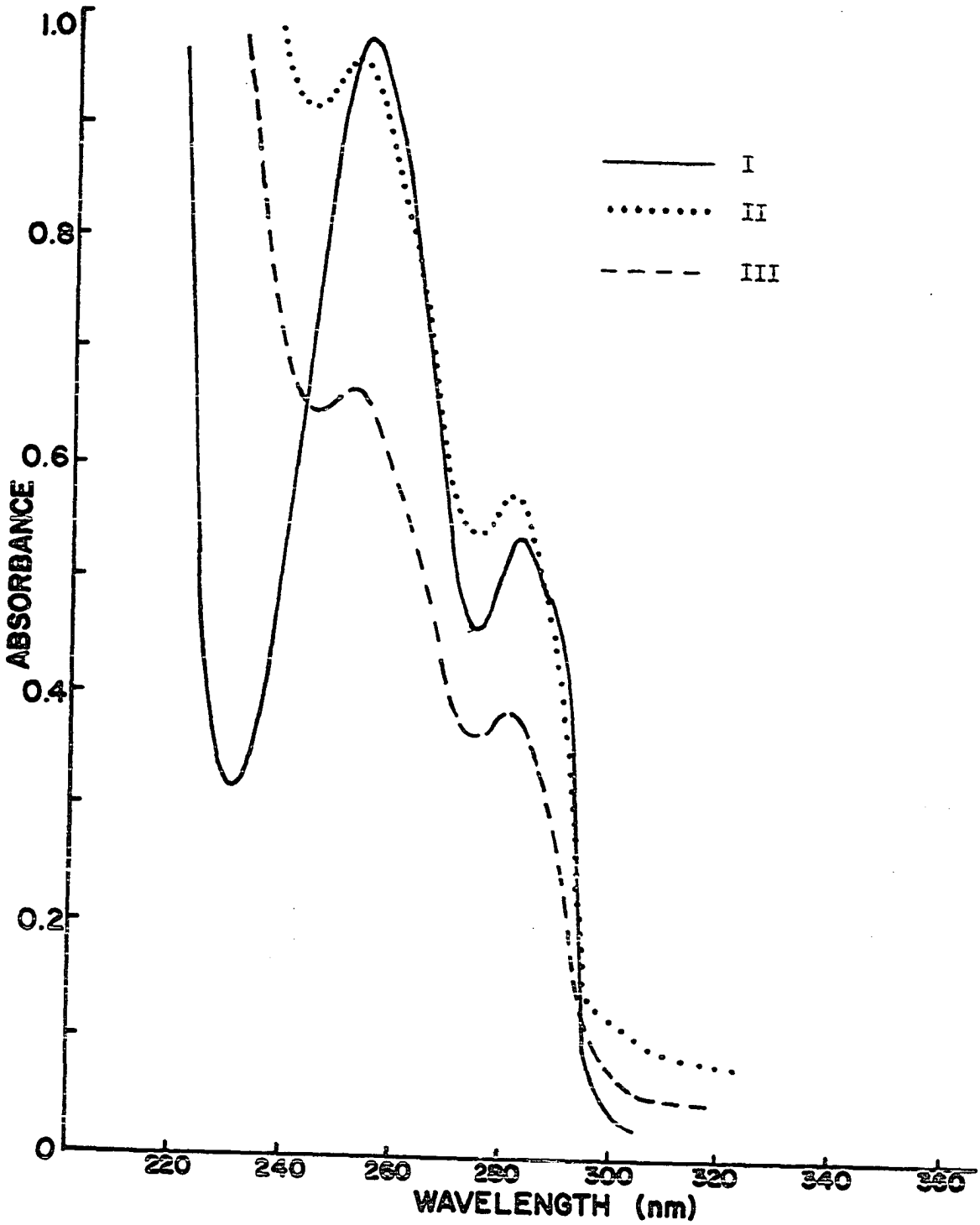

1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
220
240
260
280
300
320
340
360
WAVELENGTH (nm)
I
II
III

Figure 23. The change of UV spectrum of DIMBOA on silica gel TLC

I. DIMBOA

II. DIMBOA (same concentration as I) on TLC for 2 - 6 hours

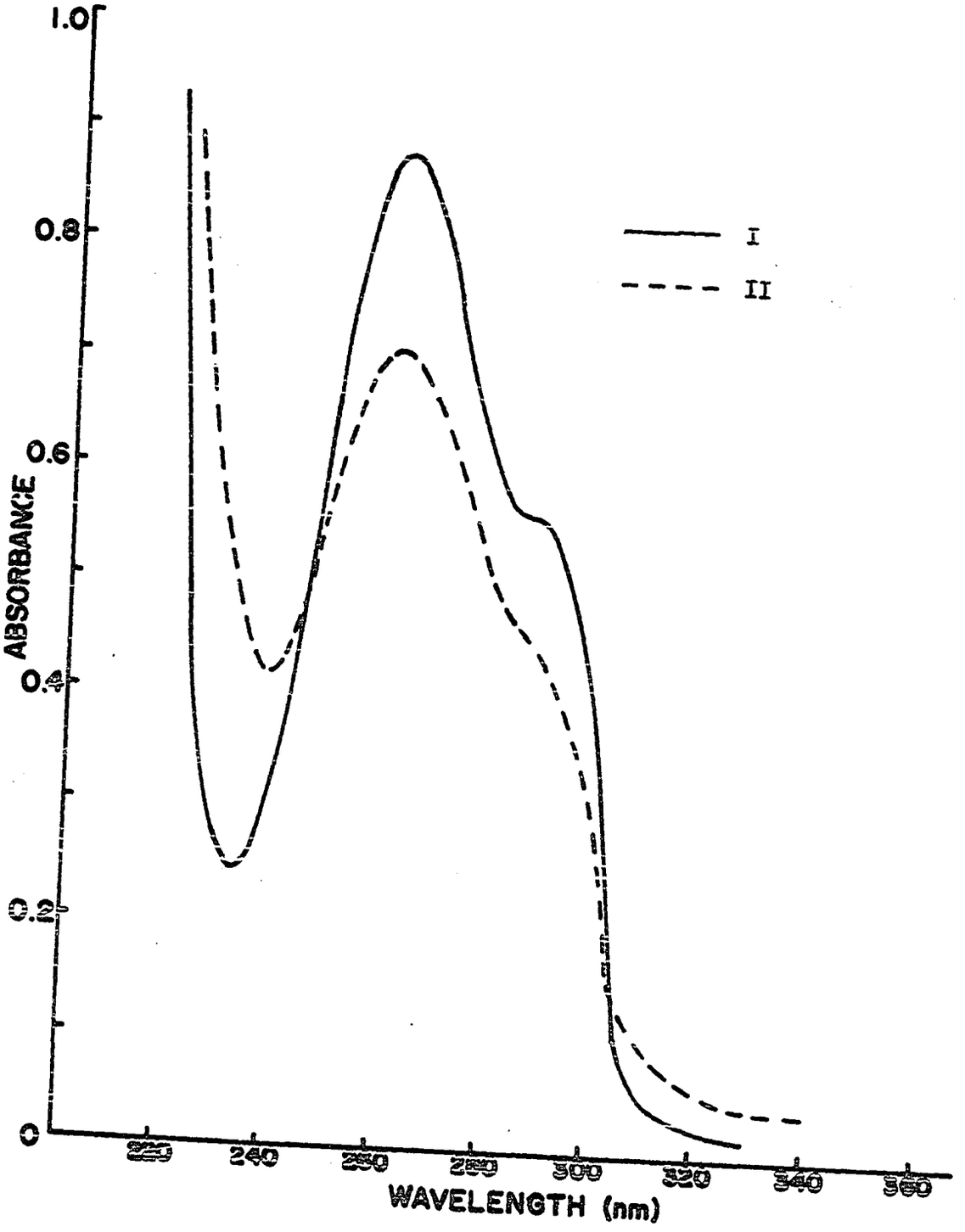
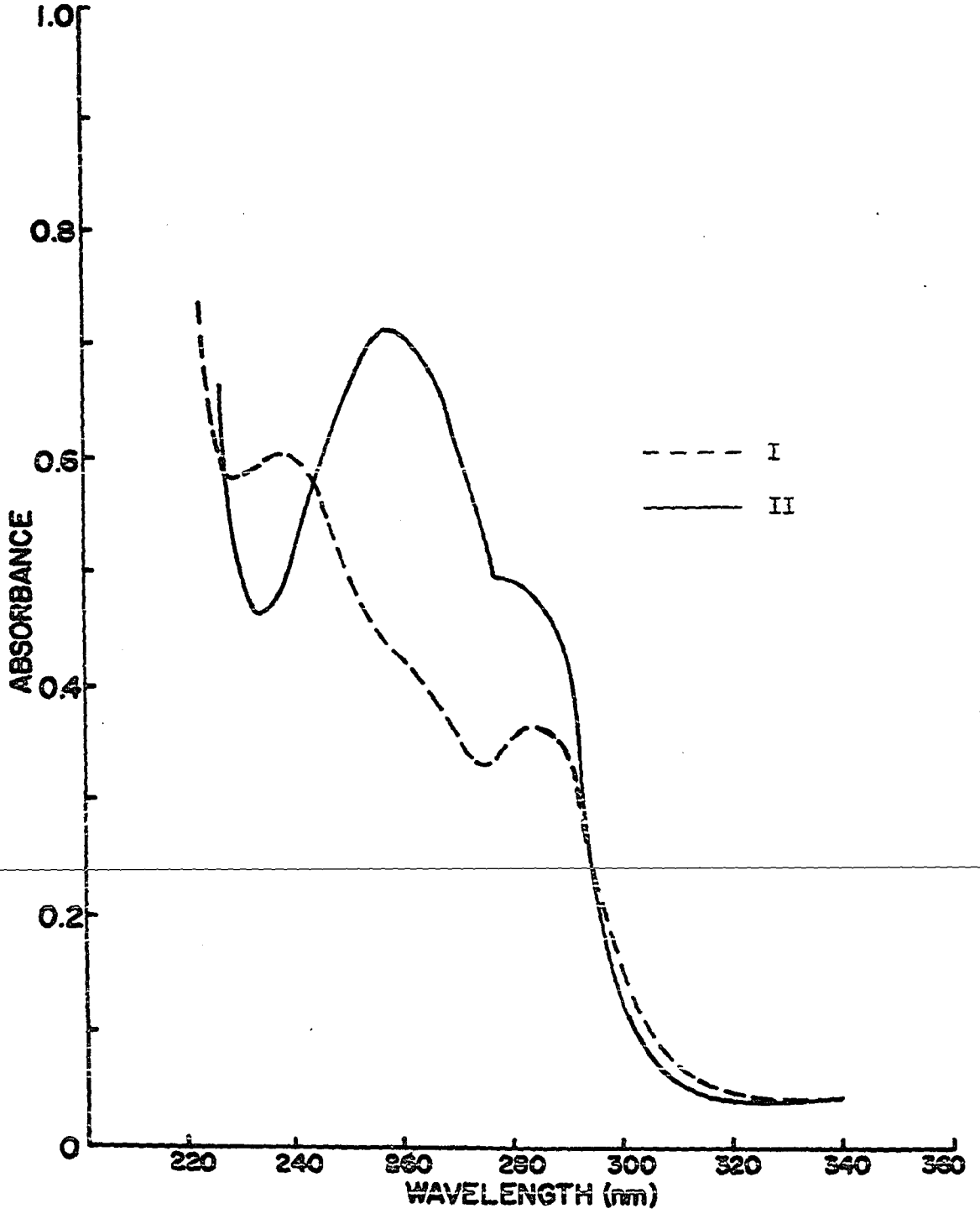

1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
I
II
220
240
260
280
300
320
340
360
WAVELENGTH (nm)

Figure 24. The UV spectrum of AHBOA

I. AHBOA in 95% ethanol

II. AHBOA (same concentration as I) in acidic ethanol


1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
220
240
260
280
300
320
340
360
WAVELENGTH (nm)
I
II

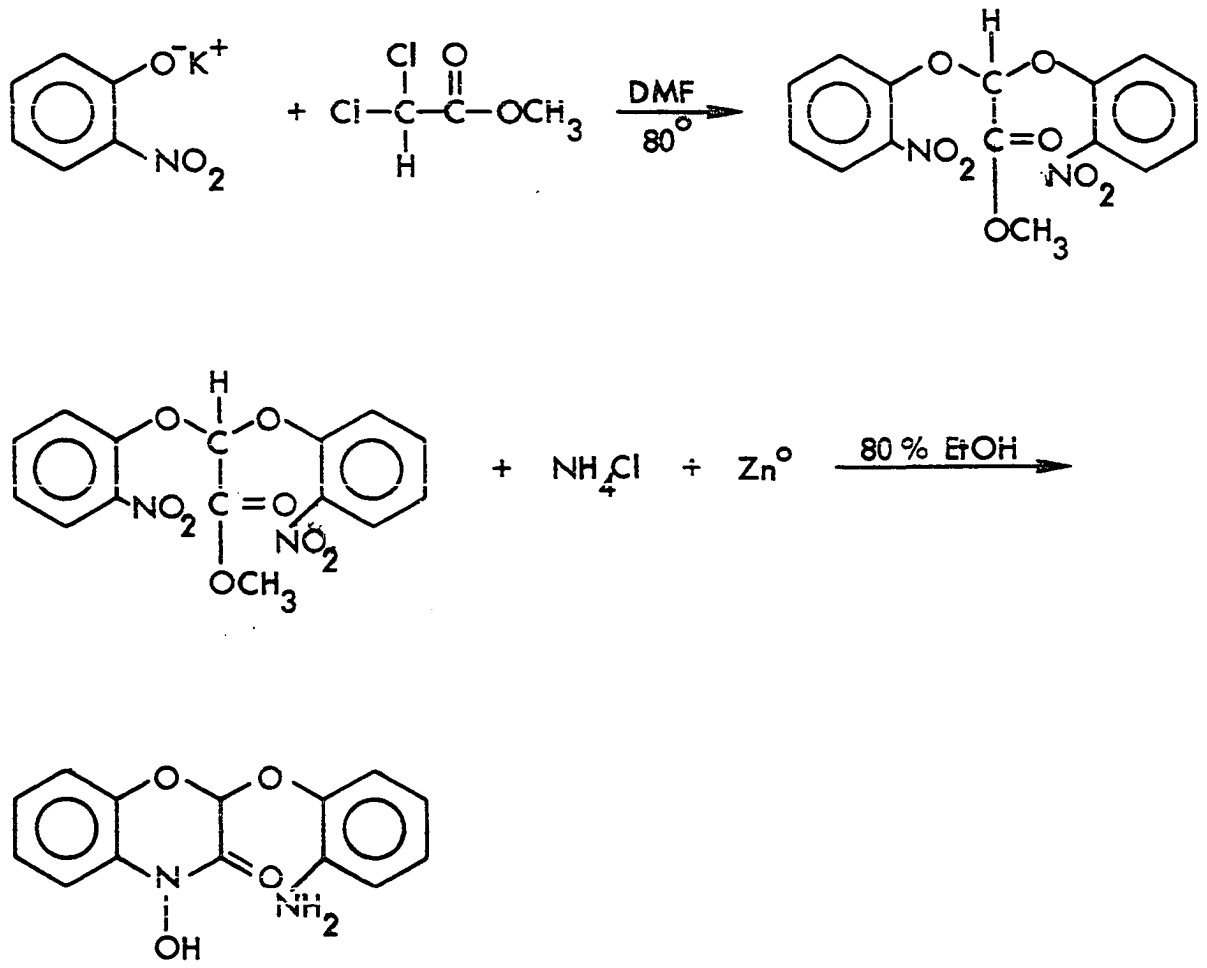


Figure 25. Synthesis of AHBOA

Efforts to hydrolyze this compound to DIBOA were unsuccessful.

A zinc chelate was isolated from the reduction of methyl bis-(o-nitrophenoxy)-acetate when treatment with EDTA was omitted. The zinc analysis shows 9.45% zinc in the chelate molecule (calculated zinc content is 10.7% based on $C_{28}H_{22}N_4O_8Z_n$). The zinc chelate structure is shown in Figure 26.

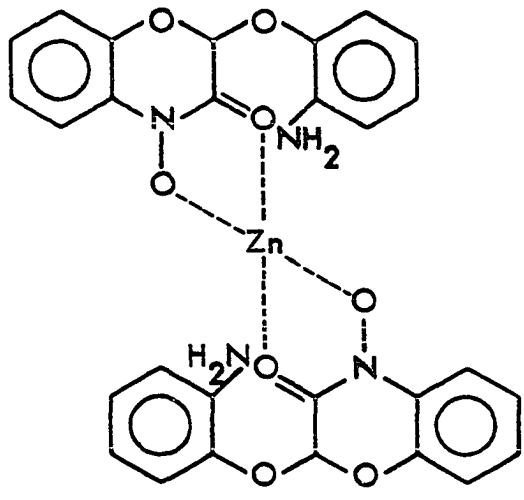


Figure 26. The structure of zinc chelate of AHBQA

DISCUSSION

Metabolism of Benzoxazinones

The study of the biosynthesis of DIMBOA was first reported by Reimann and Byerrum (34, 35). They concluded that the aromatic moiety of DIMBOA is biosynthesized from quinic acid via the shikimic acid pathway. This conclusion was supported by the observation that radicactive shikimic acid was incorporated into DIMBOA (75). Reimann and Byerrum also suggested that a nitrogen-containing aromatic product of the shikimic acid pathway could be an intermediate. Husted, using anthranilic acid-^{15}N (78) demonstrated that the amino group of anthranilic acid is the source of nitrogen for the heterocyclic ring of DIMBOA. The ^{15}N was incorporated into DIMBOA with a dilution factor of 14, but rather large dilution factors for anthranilic acid-1-^{14}C, from 280 to 7,600 in several experiments, were reported by the same author. However, the variation in the dilution factor for anthranilic acid-1-^{14}C was eliminated in the experiments reported here. The dilution factors were 49 and 48 (Table 1) for anthranilic acid-1-^{14}C in two experiments when the same amount of anthranilic acid-1-^{14}C and same age, same number of corn seedlings were used for the feeding experiment. This result also confirms Husted's (78) conclusion that anthranilic acid is one of the intermediates between shikimic acid and DIMBOA.

The dilution factor is affected by several parameters in addition to those, e.g., the amount of labeled material fed and the number, size and age of the plant, mentioned above. Others include the endogenous pool size of the material fed, the end product, and the intermediates, and the rate

of conversion of precursor to product. Therefore, when two labeled materials are fed to investigate the precursor-product relationship, the experimental conditions should be as nearly the same as possible.

HMBOA in Table 1 has the specific activity 378 cpm/μmole with a dilution factor of 101 for anthranilic acid-1-^{14}C. It is not possible to determine the metabolic relationship between DIMBOA and HMBOA with the present result. Wang (17), using radioactive DIMBOA-glucoside, HMBOA-glucoside, DIMBOA, and HMBOA, suggested that DIMBOA and HMBOA as well as DIMBOA-glucoside and HMBOA-glucoside are interconvertible, but his experiments did not clarify the question whether the interconversion takes place before or after hydrolysis of the glucosides.

In Tecoma stans, 3-hydroxyanthranilic acid and o-aminophenol are intermediates in the biosynthesis of catechol (51) and isophenoxazine (53) from anthranilic acid. 3-Hydroxyanthranilic acid was found not to be a precursor of DIMBOA (75). The result of the feeding of o-aminophenol-^{14}C in Table 1 shows that this compound is not incorporated into DIMBOA at all. o-Aminophenol is rather unstable towards air oxidation, therefore, L-ascorbic acid was added to the feeding solution to prevent the air oxidation of o-aminophenol. o-Aminophenol can be oxidized by phenol oxidase or peroxidases (79). Phenylhydrazine, which was shown to inhibit, specifically and irreversibly, a number of catechol oxidases from plant tissues (80), was added in some experiments to prevent enzymatic oxidation of o-aminophenol. The phenyhydrazine did not interfere with incorporation of anthranilic acid-^{14}C into DIMBOA, but there was still no incorporation of o-aminophenol. Therefore, o-aminophenol cannot be an intermediate between anthranilic acid and DIMBOA.

Reimann and Byerrum (34) postulated that the N-deoxyribulotide of an aromatic amine derived from the shikimic acid pathway is an intermediate in the biosynthesis of DIMBOA. They also reported that D-ribose-1-^{14}C is incorporated into DIMBOA specifically at the C-3 atom (62.5%). Therefore, the N-deoxyribulotide was suggested to be the product from anthranilic acid and ribose phosphate (79). Since phosphate esters are not readily taken up by intact plant cells, a cell free system was devised by Tu (75) to study the biosynthesis of DIMBOA from N-deoxyribulotides and some other precursors, e.g., anthranilic acid, 3-hydroxyanthranilic acid, and o-aminophenol. She proposed that the ascorbate which has been added in the preparation of the cell-free extract is the immediate precursor for the formation of the oxazine ring of DIMBOA. As postulated by Tu, if ascorbate is the immediate precursor of DIMBOA, then the ^{14}C incorporation from L-ascorbic acid-1-^{14}C into DIMBOA should be predominantly at carbon 3 atom of the DIMBOA molecule. The results in Table 1 show that the label is only 1.5% at carbon 3 and 5% at carbon 2 while D-ribose-1-^{14}C has 40% incorporation at carbon 3 and 6% at carbon 2 of DIMBOA. This result supports Reimann and Byerrum's (34) report that ribose was the precursor whose carbons 1 and 2 contributed the carbons 3 and 2 of the oxazine ring of DIMBOA. The significant specific activity of the aromatic moiety of DIMBOA from the feeding experiments with D-ribose-1-^{14}C and L-ascorbic acid-1-^{14}C was not measured accurately due to the strong quenching by the intense brown color from 5-methoxy-o-aminophenol.

Tu reported earlier (75) that anthranilic acid-1-^{14}C, o-aminophenol-^{14}C and 3-hydroxyanthranilic acid-^{3}H, as well as the deoxyribulotides of these compounds, were incorporated into DIMBOA by her cell-free system.

Since DIMBOA is quite unstable toward silicic acid (Figure 23) and no other chromatographic system for its purification is available, she purified her product by repeated crystallization. In repeating her work with anthranilic acid-1-^{14}C and o-aminophenol-^{14}C the partially purified DIMBOA was degraded to 6MBOA in hot water and the 6MBOA was then purified by TLC. Using this procedure, no labeling of 6MBOA was observed. Anthranilic acid can form a nonenzymatic product with DIMBOA which has an Rf value very close to that of DIMBOA in the TLC system used here (Figures 4, 5). The radioactivity found in DIMBOA by Tu was almost certainly due to contamination by this or a similar product.

One of the factors which can make plant proteins particularly unstable and difficult to work with is the presence of phenolic compounds. These compounds can be oxidized enzymatically or nonenzymatically to give quinones which will inhibit many enzymes and subcellular organelles (79). This inhibition can be prevented by thiols and other reducing agents (81). As mentioned in the Experimental Section of this work, ascorbic acid and mercaptoethanol were used as the reducing agents to prevent the oxidation of phenolic compounds in the cell-free extract (E-1). The phenolic compounds were also removed by Sephadex G-25 Column (E-2) or by preparing an acetone powder (E-3). None of these preparations catalyzed DIMBOA synthesis. The activity of anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase in the cell-free extract (E-1) has been investigated by measuring the change of fluorescence of anthranilic acid as described by Smith and Yanofsky (82). No enzymatic activity was observed. Two major factors which could cause the cell-free system to be inactive toward anthranilic acid are suggested. One reason

could be that some co-factors required for the activity of the enzymes have been dissociated from the proteins in the cell free extracts. The second reason could be that the enzymes for DIMBOA synthesis are in a multienzyme complex from which intermediates do not dissociate and which does not combine with added intermediates.

All results from the feeding experiments of earlier reports and of the present work show that anthranilic acid is unlikely to be converted to another aromatic amine, e.g., 3-hydroxyanthranilic acid or o-aminophenol, before reacting with a ribose phosphate. This suggests that the next intermediate is N-5'-phosphoribosyl anthranilate, also an intermediate in tryptophan biosynthesis. Since no significant amount of radioactive protein was obtained from the feeding experiment with anthranilic acid-1-^{14}C, tryptophan is probably not formed from added anthranilic acid in the young corn seedlings.

The study of the fixation of radioactive CO_2 into DIMBOA by seedlings of four inbred varieties of corn, B49, B52, CI31A and WF9, shows that these four varieties can be segregated into two groups, B49 and CI31A have slower degradation rates (Table 2), higher specific activity (Table 3) and require a longer time to reach the maximum specific activity of 6MBOA (Figures 7-10). It has been observed in this experiment that the content of DIMBOA remained nearly constant in the plant during the period of the experiment. Obviously, the change of the specific activity of 6MBOA is not affected significantly by a change in the concentration of DIMBOA.

Klun and Robinson (25) have pointed out that the resistance of corn to the 1st-brood European corn borer is directly related to the concentration of DIMBOA. They observed that the young seedlings of the four

inbred varieties, B49, B52, CI31A and WF9 have higher concentration of DIMBOA than at the whorl stage of older plants, and all these four young inbred varieties have apparent resistance to the European corn borer. However, only B49 and CI31A, that maintained high concentrations of DIMBOA in the whorl tissue at later stages of development, were borer resistant in the field. At the whorl stage, B52 has only intermediate resistance to the borer whereas WF9 is susceptible, Klun and Robinson (25) concluded that the change of the resistance to the 1st-brood European corn borer of the four inbred varieties could be attributed to the change of the concentration of DIMBOA or the content of the glucoside precursor.

These experiments show that the change of the concentration of DIMBOA is controlled by the degradation rate of this compound. In other words, from the rate of the degradation of DIMBOA in seedlings, it is possible to predict the resistance to the 1st-brood European corn borer. Table 7 shows the correlation between the degradation rate of DIMBOA, the concentration of 6MBOA in seedlings, the resistance rating and the concentration of 6MBOA at the whorl stage in four inbred varieties, B49, B52, CI31A and WF9. The correlation in Table 7 shows, generally, the inbred variety which has the slowest degradation rate has the highest resistance and highest 6MBOA concentration.

Table 3 shows that the maximum specific activities of 6MBOA in B49 and CI31A are higher than in B52 and WF9. This cannot be explained by dilution by pre-existing DIMBOA but it could be explained if the pool size of the intermediates, e.g., in WF9, is larger than that of B49. Again, if a higher percentage of the intermediate in B49 is converted to DIMBOA than in WF9, a high specific activity and higher content of 6MBOA in B49 should

Table 7. The correlation between the degradation rate, concentration of 6MBOA in seedling, resistance rating and the concentration of 6MBOA at the whorl tissue at different height of the plant in four inbred lines

Imbred variety	Degradation rate[a] k (hr^{-1})	6MBOA[b,c] Plant height (in.) 6	18	33	Resistance rating[d]
CI31A	1.74×10^{-2}	33.3	23.89	12.18	1.95
B49	5.07×10^{-2}	54.5	15.03[e]	13.73	2.10
B52	9.57×10^{-2}		6.18	3.51	5.66
WF9	8.85×10^{-2}	42.4	2.97	1.69	7.95

[a]From Table 2.

[b]μmole/g dry tissue.

[c]Klun and Robinson (25), Figure 5 and Table 4.

[d]Klun and Brindley (26) (the small rating, the higher resistance to borers; the larger rating, the higher susceptibility to borers).

[e]At 15 in.

be observed.

The specific activities of DIMBOA and HMBOA rise and fall roughly in parallel following $^{14}CO_2$ administration (Figures7-10). This is consistent with the suggestion of Wang (17) that they have a common precursor and are interconverted, rather than being precursor and product.

In the CO_2 fixation experiments, it was concluded that the degradation rate is one of the factors controlling the content of DIMBOA in the corn plant. Wang (17) has observed an enzyme, 2-hydroxy-benzoxazinone reductase, which he suggested might be involved in the degradation of the 1,4-benzoxazinones. The results in Table 8 are the conversion of the previous data and

show that there is not a close correlation between the activity of this enzyme and the rate of turnover of DIMBOA. However, since the activity of this enzyme is of about an order of magnitude less than the degradation rates observed in CO_2 fixation, this enzymatic reaction is unlikely to play a role in the control of the content of the benzoxazinones in the corn plant.

The enzymatic hydrolysis of benzoxazinone glucosides to the aglucone catalyzed by β-glucosidase is an important step in the degradation of the glucosides. Results in Table 8 show that this enzymatic hydrolysis is probably not rate controlling because of high activity compared to low turnover rate; differences between varieties are not large. There may be a specific β-glucosidease for benzoxazinone glucosides but since a synthetic substrate was used for these assays it could not be differentiated if present.

Table 8. Rate of degradation of benzoxazinones and the glucosides

Inbred variety	DIMBOA degraded[a] μmole/24 hr/ g dry tissue	2-Hydroxy-benzoxazinone[b] reductase μmole/24 hr/ g seedling	β-glucosidase[c] μmole/24 hr/ g seedling
WF9	56.40	0.37	2.30×10^3
B49	46.56	0.17	1.87×10^3
CI31A	10.08	0.34	1.96×10^3

[a]Calculated from the concentration of 6MBOA/g dry tissue at 6" in Table 7 and the half-time in Table 2.

[b]From Table 4.

[c]From Table 5.

Chemical Aspects of Benzoxazinones

Hamilton and Moreland (63) reported that DIMBOA was catalyst for the hydrolysis of simazine to hydroxysimazine. Husted (78) characterized this reaction further and showed that the free acid form, but not the anion, of DIMBOA is catalytically active. The rate of simazine hydrolysis increases more than 10-fold when the DIMBOA concentration is increased 10-fold. This, along with a small but significant decrease in the extinction coefficient of DIMBOA as the concentration increases, suggest that molecular aggregates may be involved in the catalysis (65).

It has been mentioned that the DIMBOA and HMBOA are interconvertible *in vivo* (17). The enzymatic interconversion has not been demonstrated *in vitro*, perhaps because of the high activity of β-glucosidase, (since benzoxazinone glucosides may be the substrates for the enzymatic interconversion). A study of the degradation of DIMBOA in acid solution was undertaken because acknowledge of the nonenzymatic reactions may suggest possible pathways of enzymatic reaction. Coutts and Pound (69) have indicated that para-chlorine-substituted amines are the major products for the aromatic hydroxamic acids in hydrochloric acid, with an imide intermediate suggested. Three products have been isolated from the degradation of DIMBOA in hydrochloric acid solution: 6MBOA, 5-methoxy-*o*-aminophenol and a chlorine-containing material of unknown structure. The mechanism of their formation is suggested according to Coutts and Pound's observation as seen in Figure 27. An oxonium ion is formed instead of para-substitution of the aromatic ring. The next compound is an *o*-quinoneimide which is a common intermediate for 6MBOA and 5-methoxy-*o*-aminophenol. The precursor of 5-methoxy-*o*-aminophenol was suggested to be 2-hydroxy-7-

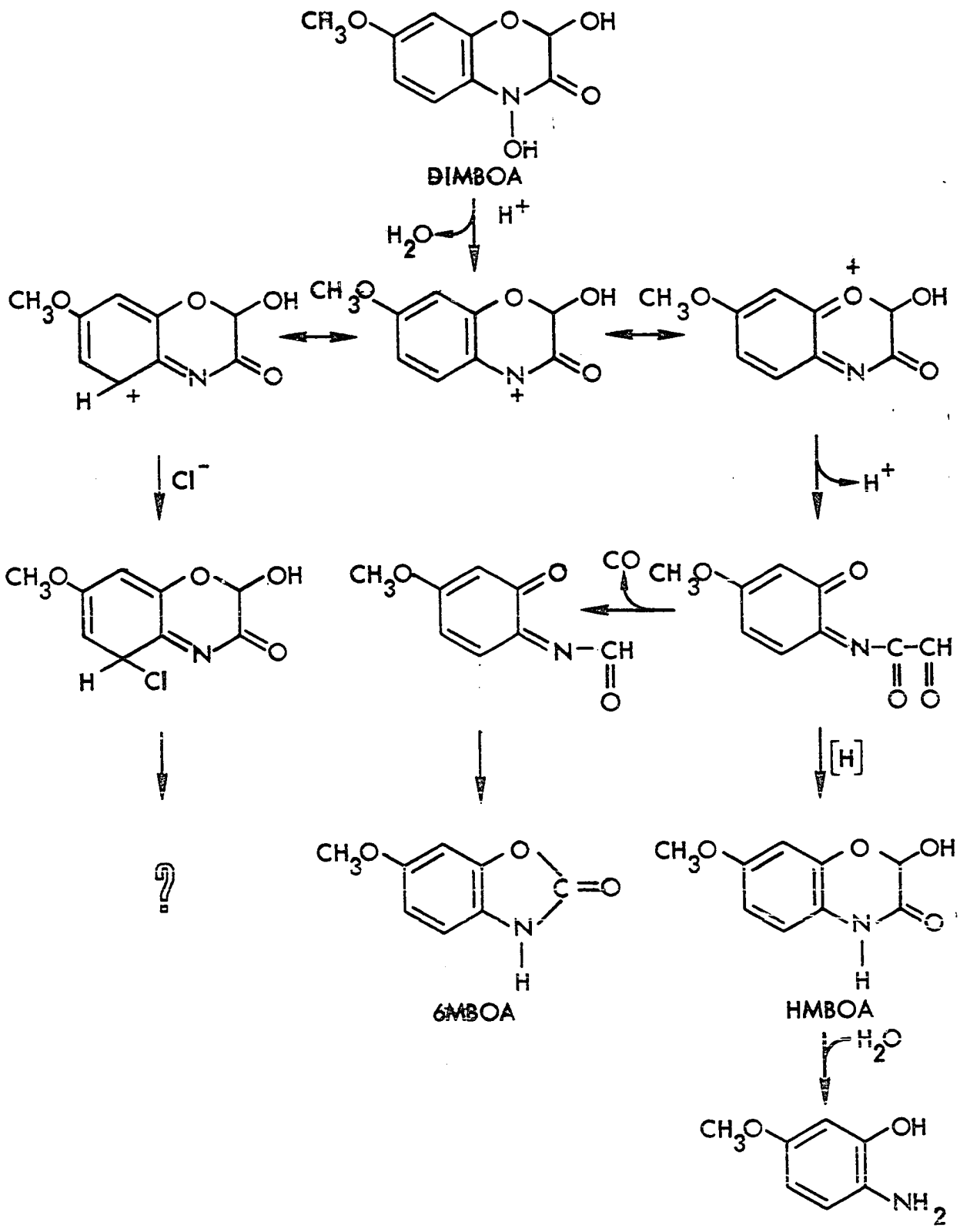


Figure 27. Degradation of DIMBOA in 1 N HCl

methoxy-1,4-benzoxazin-3-one, HMBOA, which is formed by reduction and cyclization of the o-quinoneimide and has not been isolated in this experiment. However, this suggestion was supported by the observation that HBOA is converted to o-aminophenol in 1N HCl. o-Aminophenol, or 5-methoxy-o-aminophenol is simply formed by acid hydrolysis of the lactam. 6MBOA is formed from the o-quinoneimide via a reduction-oxidation reaction after liberation of carbon monoxide. This mechanism is different from the base-catalyzed degradation in which formic acid is liberated (11). The mechanism for the formation of the lactam from the cyclic hydroxamic acid in acid solution suggests that the enzymatic conversion of the cyclic hydroxamic acid to the lactam could be an acid-catalyzed type reaction with an o-quinoneimide serving as an intermediate.

The HCl-catalyzed degradation of DIBOA has not been investigated, but one of the major products is expected to be the para-chlorine-subsituted lactam according to Coutts and Pound (69). In the enzymatic reaction, a hydroxyl group, rather than chlorine, could function as the nucleophilic reagent and substitute at the para-position; subsequent methylation would form HMBOA as shown in Figure 28. Thus, DIMBOA might be formed from DIBOA via HMBOA.

It has been mentioned in the Review of Literature that members of a series of cyclic hydroxamic acids have antifungal activity and one of them, DIMBOA, is a feeding deterrent for larvae of the European corn borer. It has also been mentioned in this Discussion that the resistance of corn to European corn borer is dependent on the content of DIMBOA in the plant. A high content of cyclic hydroxamic acid has also been reported to be related to resistance to several fungal diseases (14, 33).

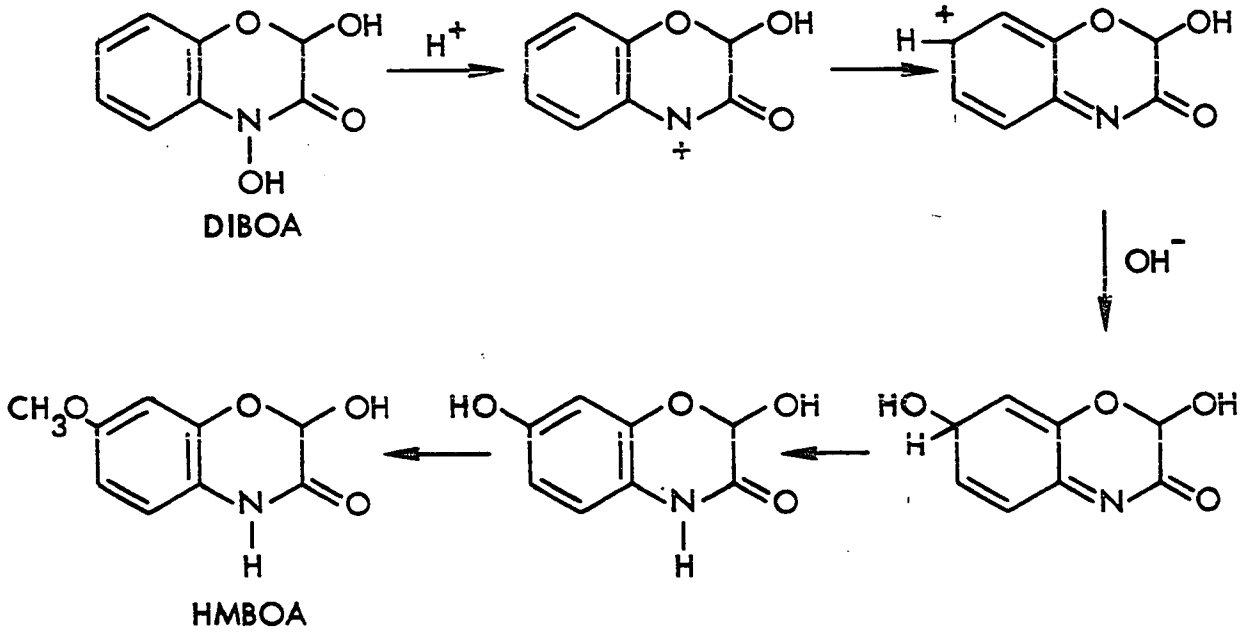


Figure 28. Hypothetic enzymatic formation of HMBOA from DIBOA

These compounds can be isolated in small quantities from the plants in which they occur but a large-scale source is not known. A route of chemical synthesis of DIBOA, used to prove the structure of this compound, was reported by Honkanen and Virtanen in 1960 (66). The overall yield of DIBOA was only 3.5%, while the related compounds, benzoxazolinone (83) and 2-hydroxy-1,4-benzoxazin-3-one (84) were synthesized in high yield without any difficulty. Two features which affect the yield in the synthesis of the cyclic hydroxamic acids are the formation of the two hydroxyl groups, one at the nitrogen atom of the hydroxamic acid and the other one at

carbon-2.

The general procedure to synthesize N-aryl hydroxamic acids is the reduction of an aryl nitro compound to the N-aryl hydroxylamine with zinc dust in ammonium chloride solution, followed by acylation. Yields are frequently low, but they can be improved by the presence of a bulky group ortho to the nitro group. This was observed by Honkanen and Virtanen (67) in the synthesis of some derivatives of benzoxazinone with higher yield, e.g., up to 70% for 4-hydroxy-2-carboxy-1,4-benzoxazin-3-one. Reduction of various o-nitrophenylthioacetates and related compounds by means of sodium borohydride and palladium-charcoal has been reported by Coutts and Pound (69) to give derivatives of the N-hydroxy compound, although only the lactam form was obtained when an attempt was made to prepare a naphthoxazine cyclic hydroxamic acid by reducing methyl (1-nitro-2-naphthoxy)-acetate using sodium borohydride and palladium-charcoal or zinc and ammonium chloride (85). The problem which has not been solved previously is the introduction of hydroxyls at both C-2 and N-4 of the benzoxazinone in good yield.

The synthesis of DIBOA reported here has been designed so that the ortho group carries an ester function which reacts with the N-aryl hydroxylamine group. More importantly the ortho substituent has a fluorine atom situated so that its replacement by hydroxyl yields the 2-hydroxy compound. The reduction of ethyl o-nitrophenoxyfluoroacetate has been carried out by the two reduction methods mentioned above; zinc and ammonium chloride, and sodium borohydride and palladium-charcoal. The former procedure yields only one product as judged by TLC on silica gel GF_{254}. The reduction by sodium borohydride and palladium-charcoal in

aqueous solution yields three by-products in addition to the hydroxamic acid, DIBOA. Therefore, the reduction by zinc dust in ammonium chloride solution seems the best procedure to prepare DIBOA and related compounds.

The yield in the last step (reduction) is low (30%) primarily because of the insolubility of the starting material, ethyl o-nitrophenoxyfluoroacetate, in water. The use of water-alcohol mixtures results in a mixture of products which includes not only the desired product, DIBOA, but 2-ethoxy- and 2-fluoro-4-hydroxy-1,4-benzoxazin-3-one. In 1,2-dimethoxyethane and bis-(2-methoxyethyl)-ether as solvents, the 2-fluoro compound appeared to be the major product. Other solvents, e.g., dioxane, N,N-dimethylformamide, and dimethylsulfoxide, have been investigated and the major product is the 2-fluoro compound in addition to a by-product.

Although α-halo ethers are very easily hydrolyzed, the fluoro compounds are the least reactive. Ethyl o-nitrophenoxyfluoroacetate and 2-fluoro-4-hydroxy-1,4-benzoxazin-3-one are stable with respect to hydrolysis under the conditions used in this synthesis and in fact the latter compound was not hydrolyzed even by treatment with $AgNO_3$ in acetone-water mixtures. This suggests that the hydrolysis of the fluorine must occur simultaneously with the reduction of the nitro group and formation of the hydroxamic acid.

Work on the synthesis of DIBOA was initiated with the reaction of o-nitrophenolate ion and methyl dichloroacetate, which is a commercial product much cheaper than ethyl chlorofluoroacetate. The product of this reaction is methyl bis-(o-nitrophenoxy)-acetate. Reduction with zinc dust yielded 2-o-aminophenoxy-4-hydroxy-1,4(2H)-benzoxazin-3-one, which resisted all efforts to hydrolyze it to the desired product. Replacement

of both chlorines of methyl dichloroacetate occurred even when the ratio of o-nitrophenolate to methyl dichloroacetate was 1:50. A similar reaction was observed by Coutts and Hindmarsh (85).

In the reduction of aryl nitro compounds to give the cyclic hydroxamic acids, the initial product is the zinc chelate. The zinc chelate was reported to be converted into the hydroxamic acid by means of 2% acetic acid (66) or 5% hydrochloric acid (85). It was found in this work that EDTA is a better reagent for removal of the zinc from the chelate complex with a satisfactory yield of hydroxamic acid.

Honkanen and Virtanen (67) reported that DIBOA and some 1,4-benzoxazine derivatives have growth-inhibiting activity against Fusarium nivale, Staphylococus aureus, Pseudomonas fluorescens and Escherichia coli. The synthetic DIBOA has been tested by Dawe and Martenson (86) who report that it inhibits the growth of Helminthosporium maydis, the fungus which causes Southern corn leaf blight. 2-Ethoxy-4-hydroxy-1,4(2H)-benzoxazin-3-one also shows the same biological activity. Bioassay of these synthetic compounds and related compounds with the European corn borer will be carried out in the near future.

Implications for Future Work

The results of the $^{14}CO_2$ incorporation studies suggest that the content of DIMBOA in plants beyond the seedling stage depends upon the rate of degradation rather than upon the rate of synthesis. This is important, since the probability of success in a search for a mutant with an unusually low rate of degradation would be expected to be greater than for a mutant with a high rate of synthesis, since most mutants cause a loss of function. Such

a mutant might have a high level of DIMBOA.

The observation that the specific activity of DIMBOA and HMBOA after exposure to $^{14}CO_2$, was lowest in the varieties with lowest concentration of these compounds was unexpected. It suggests that these varieties must have larger pools of the precursors to DIMBOA and HMBOA, but these pools are used to a large extent for synthesis of other compounds. In particular the pool size of anthranilic acid in several varieties should be measured.

Evidence for the participation of an N-acyl o-quinoneimide in the acid-catalyzed degradation of DIMBOA means that such a compound could also be considered for a role in enzymatic reactions particularly the interconversion of DIMBOA and HMBOA.

Although DIBOA has not been shown to play an important role in the resistance of corn to the European corn borer and to fungi because it is accompanied by much larger amounts of DIMBOA, it is probable that the presence or absence of the methoxyl group has little to do with the biological activity. If this is true, DIBOA, available in large quantities at low cost, should be tested as a pesticide.

SUMMARY

Some chemical and biochemical properties of the 1,4-benzoxazinones in maize have studied.

The results of feeding experiments further support the previous observations that anthranilic acid and D-ribose are the precursors of DIMBOA. Anthranilic acid is unlikely to be converted to another aromatic amine, e.g., 3-hydroxyanthranilic acid or o-aminophenol, before reacting with a ribose phosphate. This suggests that the next intermediate is N-5'-phosphoribosyl anthranilate, also an intermediate in tryptophan biosynthesis. L-ascorbic acid, suggested to be an intermediate in the biosynthesis of DIMBOA by an earlier study, showed a random incorporation with low specific activity when L-ascorbic acid-1-^{14}C was fed to the corn seedlings.

The earlier report of synthesis of DIMBOA in a cell-free system (75) was shown to be in error. An artifact, formed by nonenzymatic reaction of DIMBOA and anthranilic acid-1-^{14}C, caused the error.

The rate of degradation of DIMBOA plays an important role in determining the concentration of DIMBOA and hence the resistance to the 1st-brood European corn borer. The inbred varieties (e.g. CI31A and B49) which have the highest resistance to the borer have the lowest rate of degradation the compound. The turnover rate of DIMBOA was of about an order of magnitude larger than the observed enzymatic reduction of HBOA catalyzed by 2-hydroxy-benzoxazinone reductase, and was small compared to the β-glucosidase activity present in leaves.

Detoxification of simazine catalyzed by DIMBOA, but not the anion was

DIMBOA concentration dependent. This reaction did not follow first-order kinetics. Molecular aggregates of DIMBOA may be involved in the catalysis.

Degradation of DIMBOA catalyzed by hydrochloric acid yielded three products: 6MBOA, 5-methoxy-o-aminophenol and a chlorine-containing material of unknown structure. An o-quinoneimide was suggested to be the common intermediate for 6MBOA and 5-methoxy-o-aminophenol. 5-Methoxy-o-aminophenol was formed from HMBOA. This reaction may serve as a model of the enzymatic interconversion of HMBOA and DIMBOA. A pathway for the enzymatic formation of DIMBOA from DIBOA via HMBOA was also suggested on the basis of this observation.

The chemical synthesis of DIBOA in moderate yield (22%) was achieved. The synthetic DIBOA and some related compounds showed biological activity in inhibiting the growth of Helminthosporium maydis. Bioassay of DIBOA with the European corn borer will be carried out in the near future.

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APPENDIX

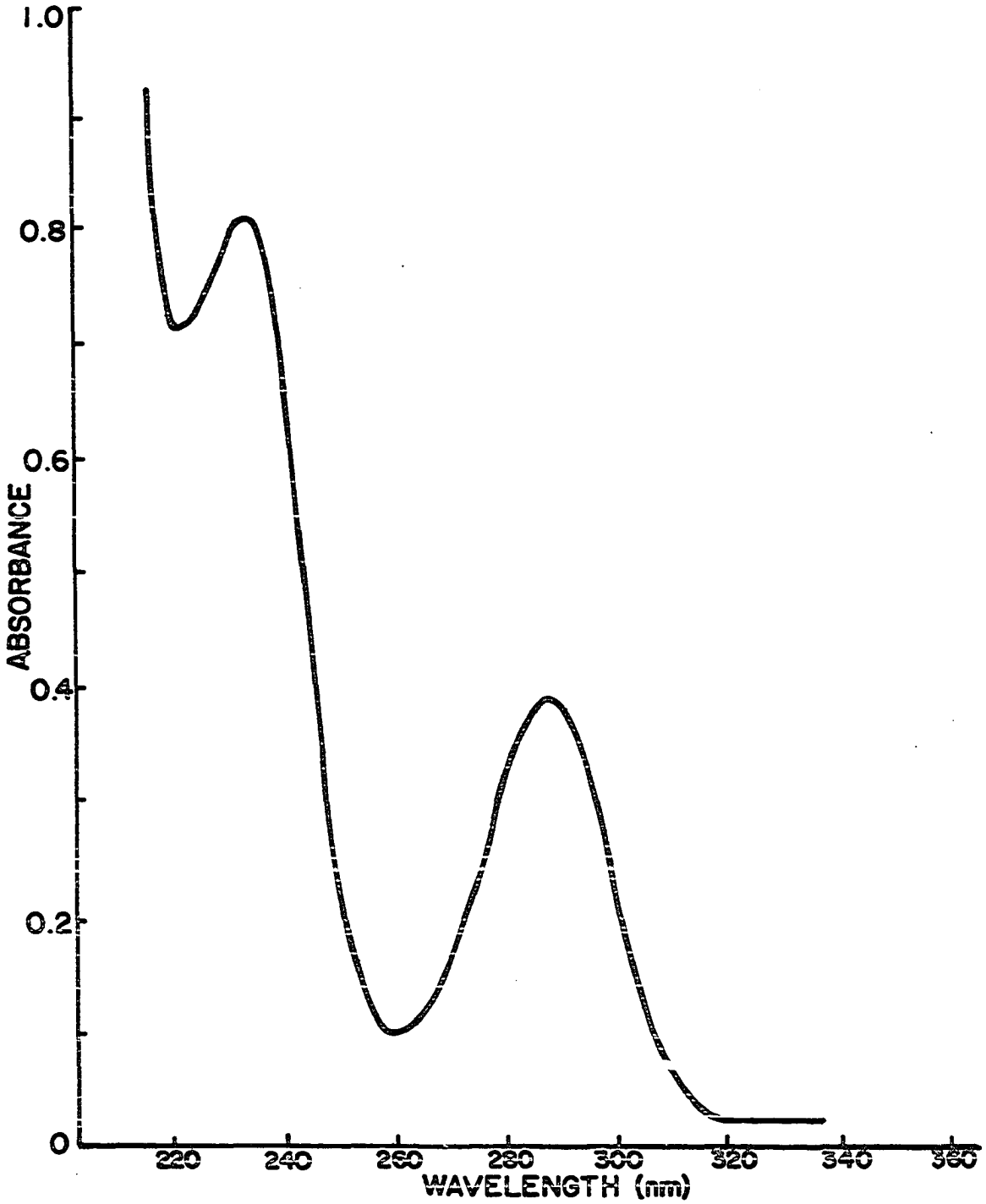

Figure A-1. o-Aminophenol

(All spectra in the Appendix were measured in 95% ethanol).

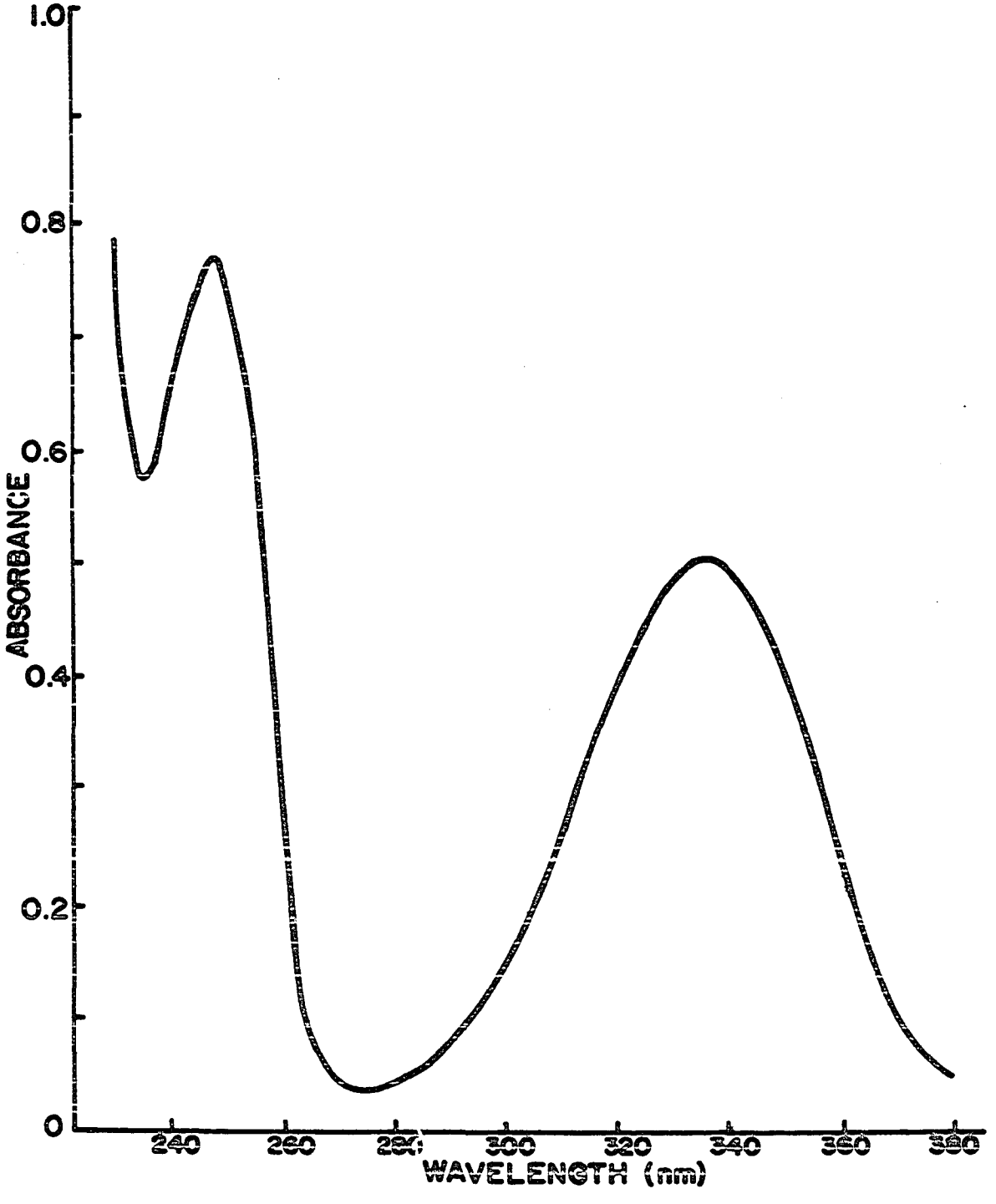

Figure A-2. Anthranilic acid

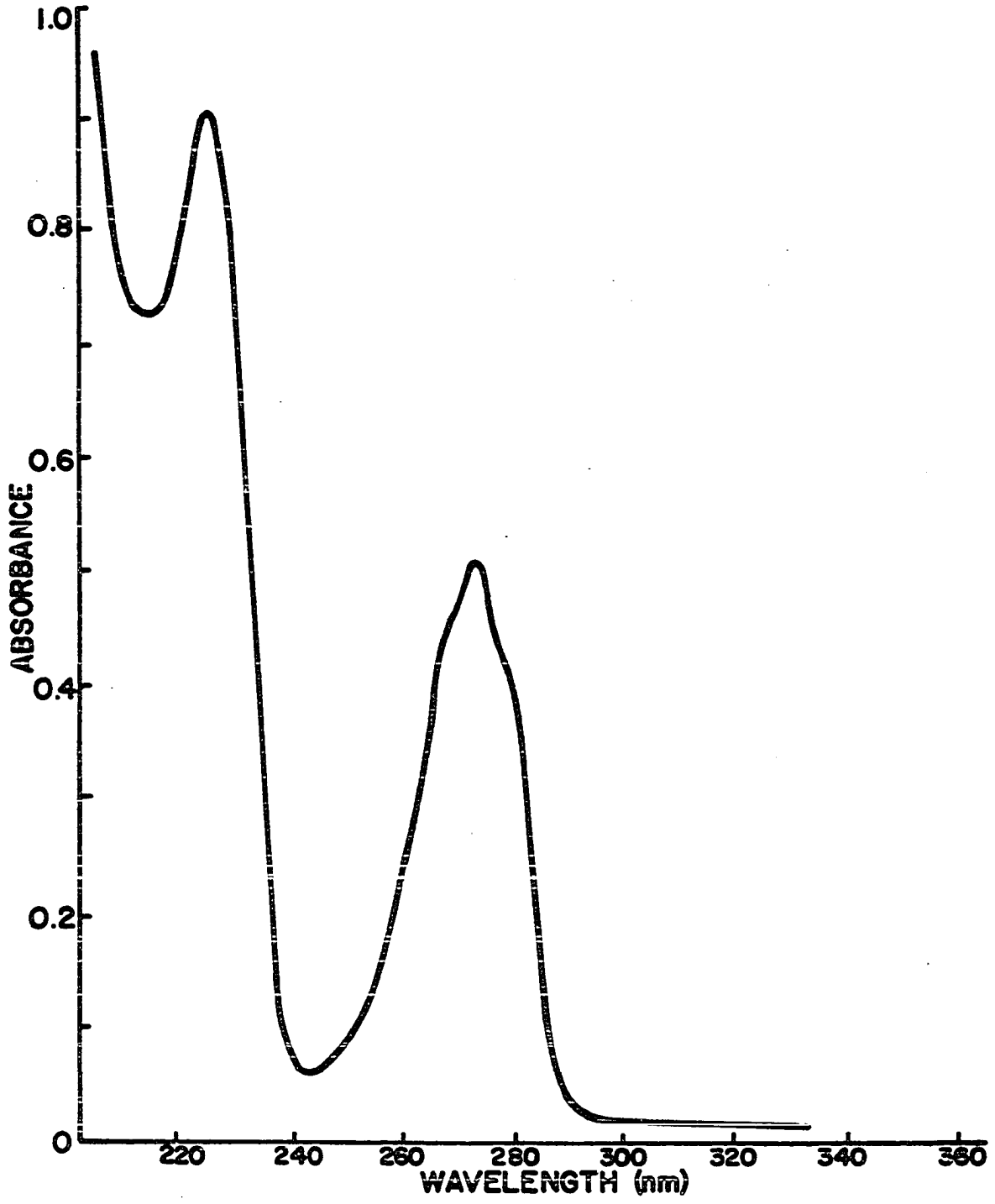

Figure A-3. BQA

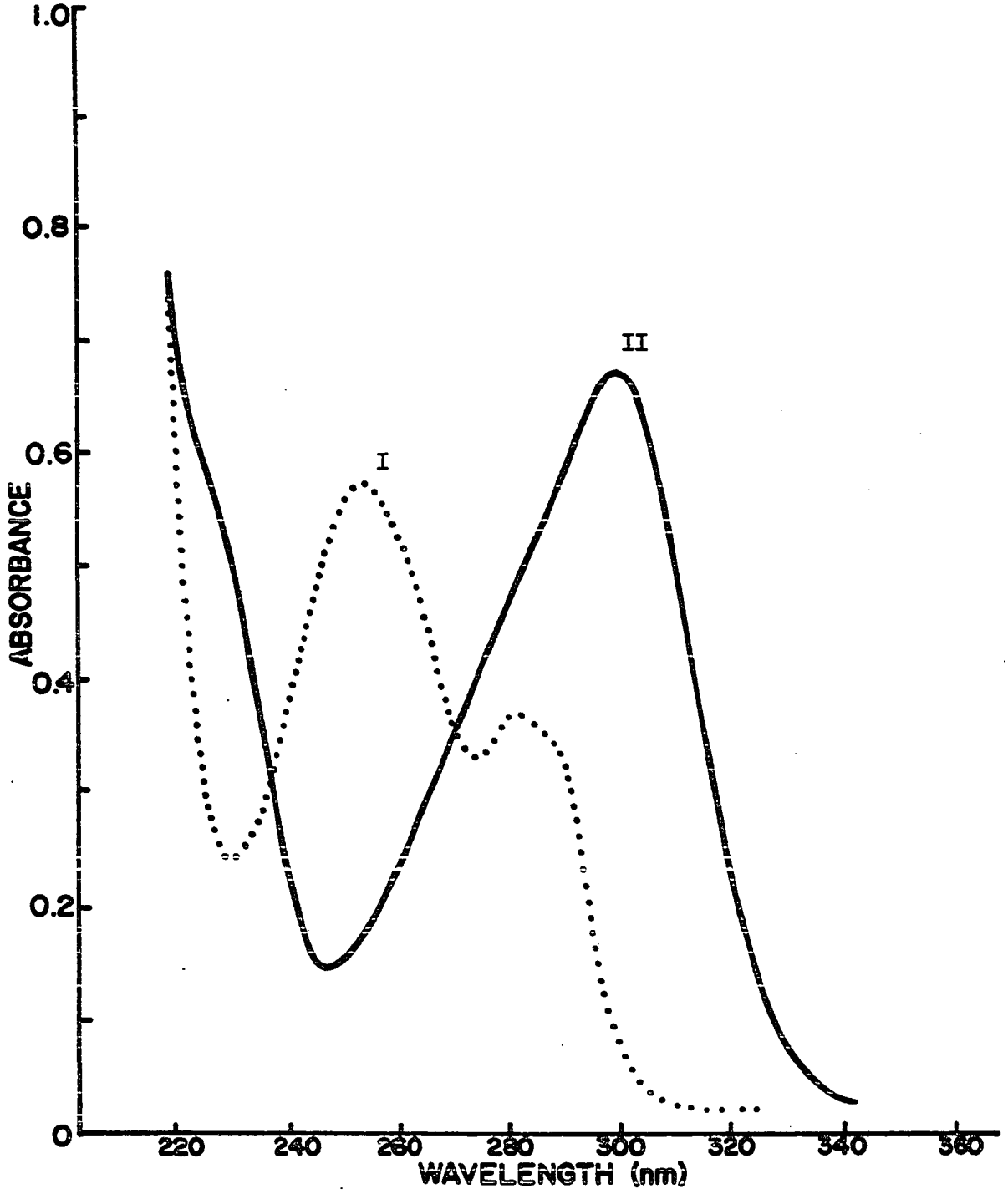

Figure A-4. I. DIBOA II. anion

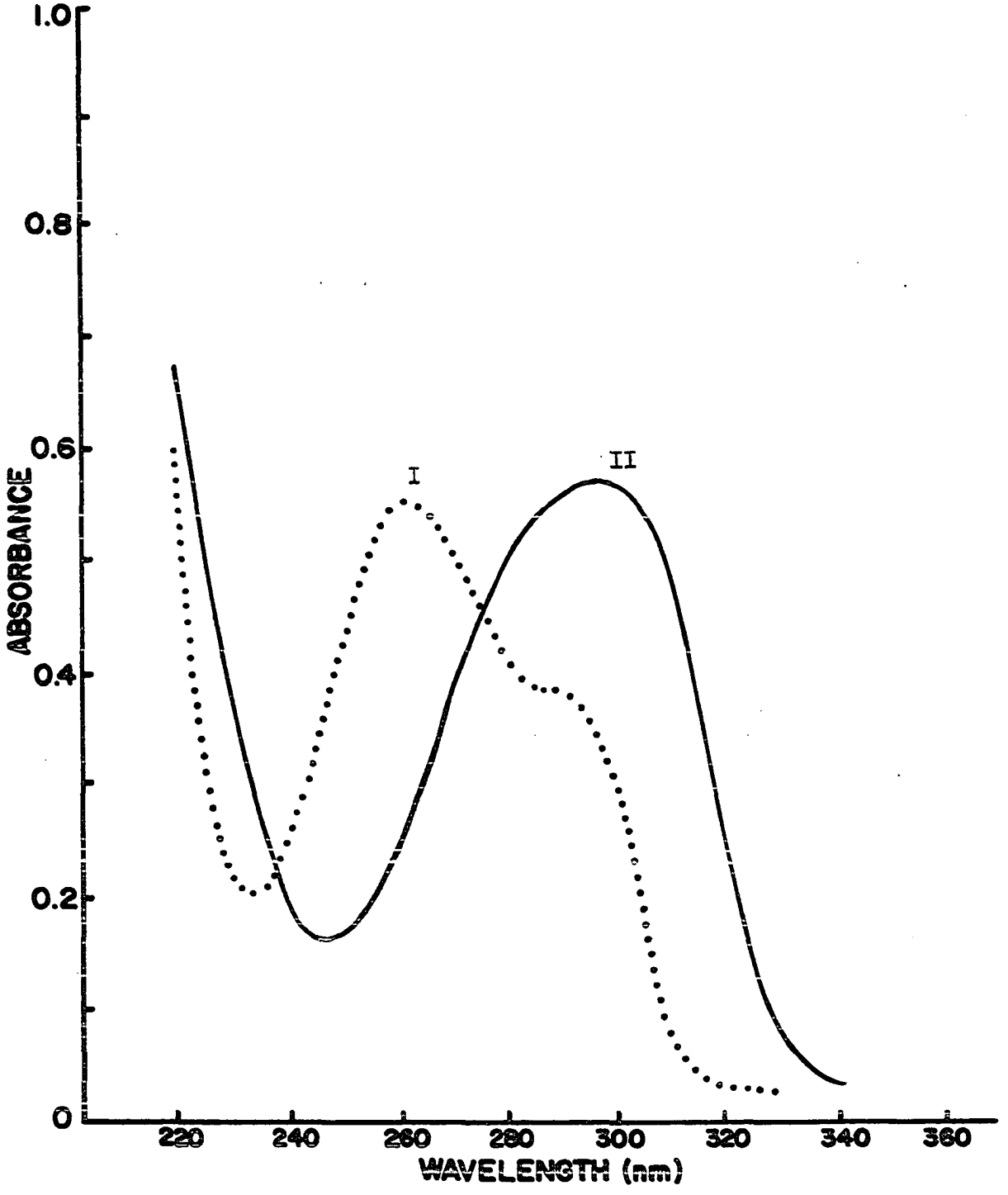

Figure A-5. I. DIMBOA II. anion

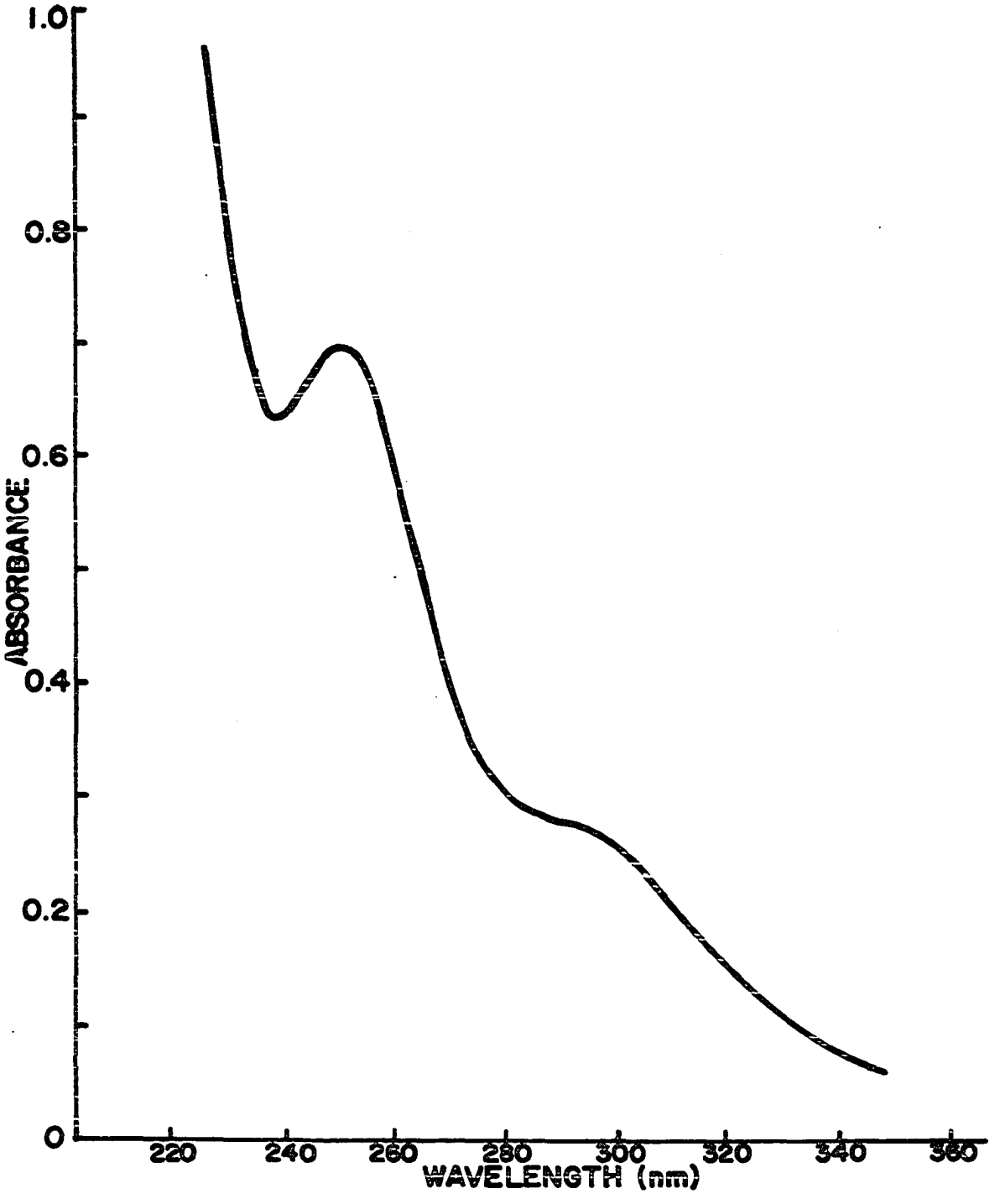

Figure A-6. Ethyl-o-nitrophenoxyfluoroacetate

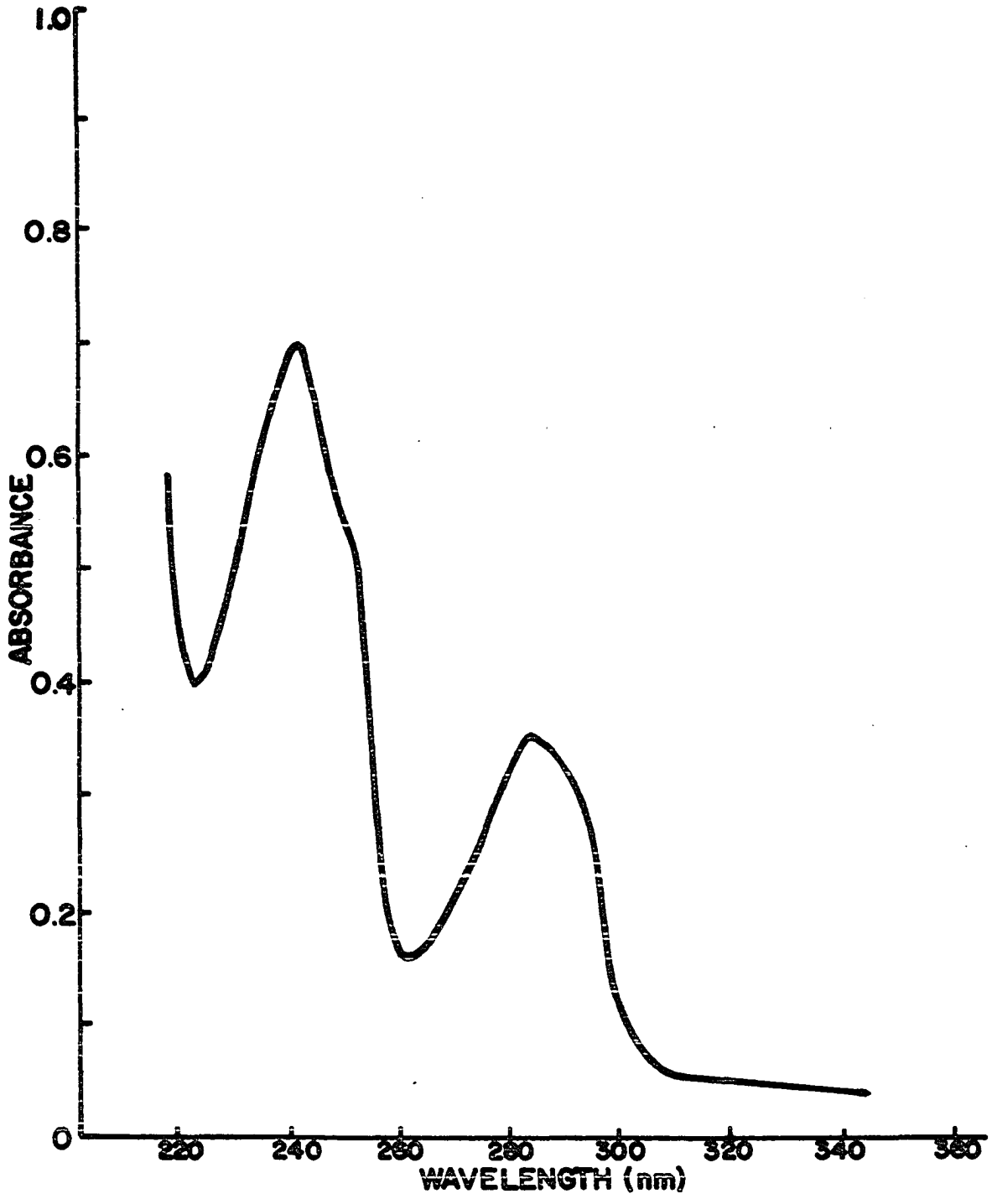

Figure A-7. GAP

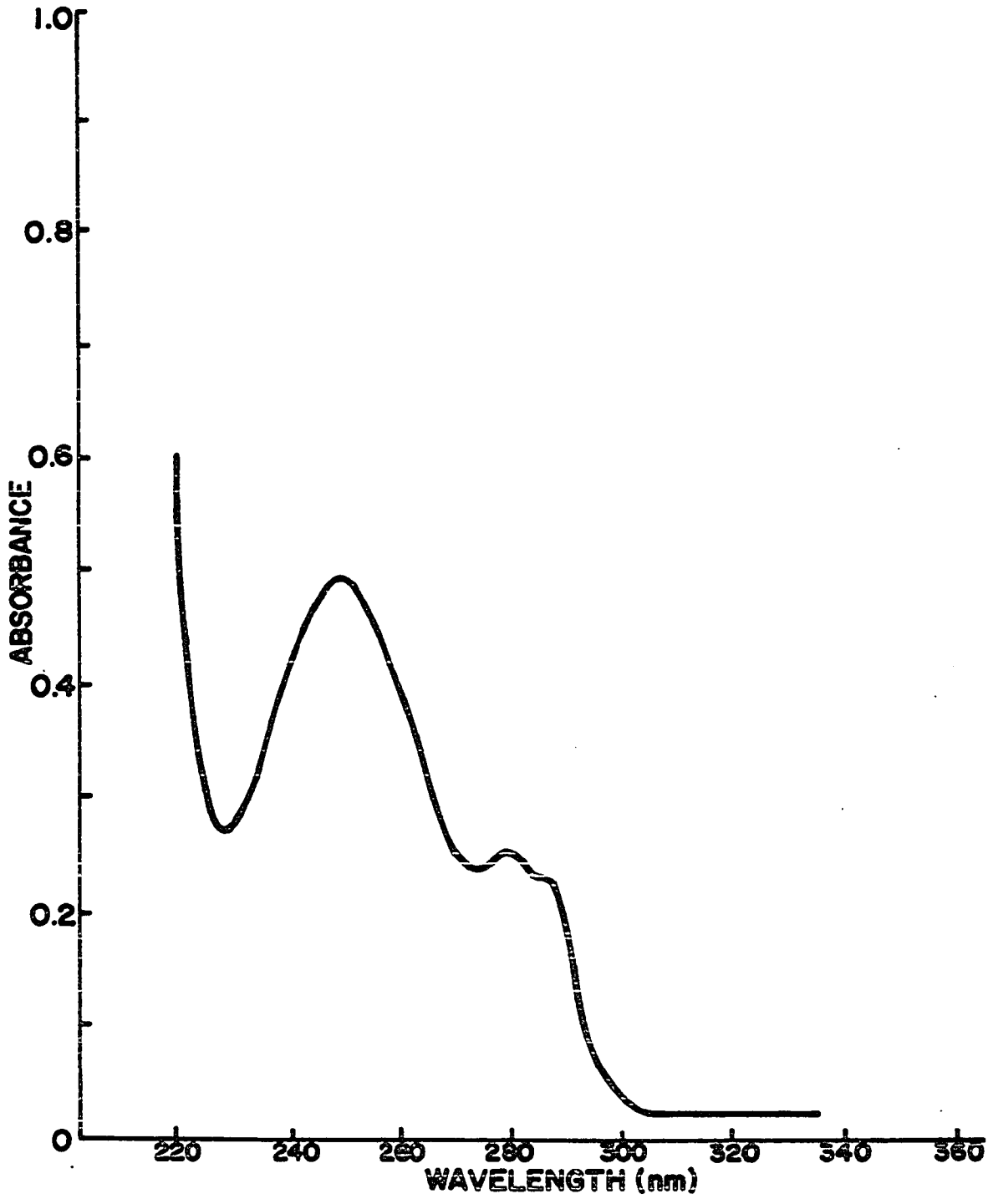

Figure A-8. HBCA

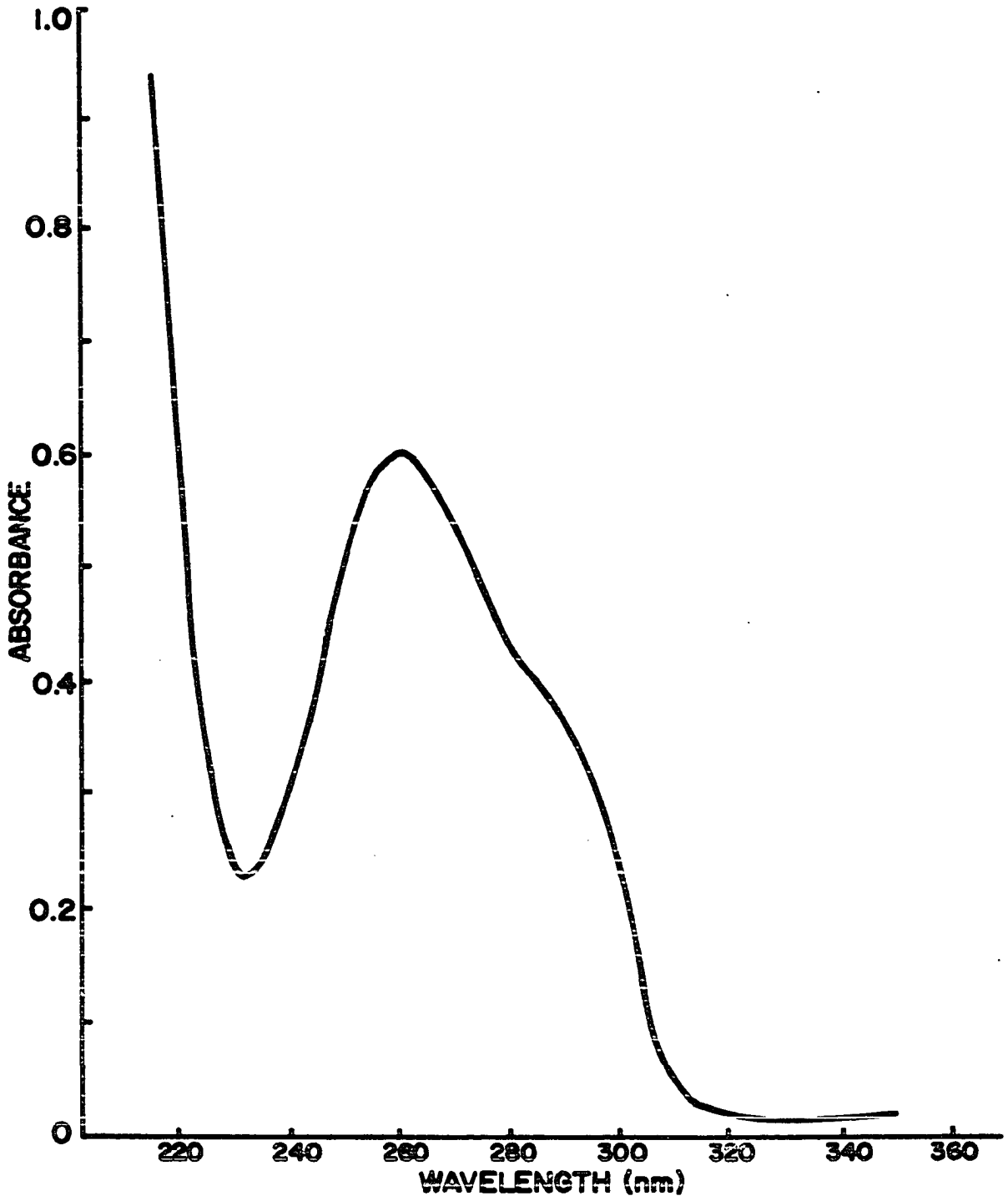

Figure A-9. HMBOA

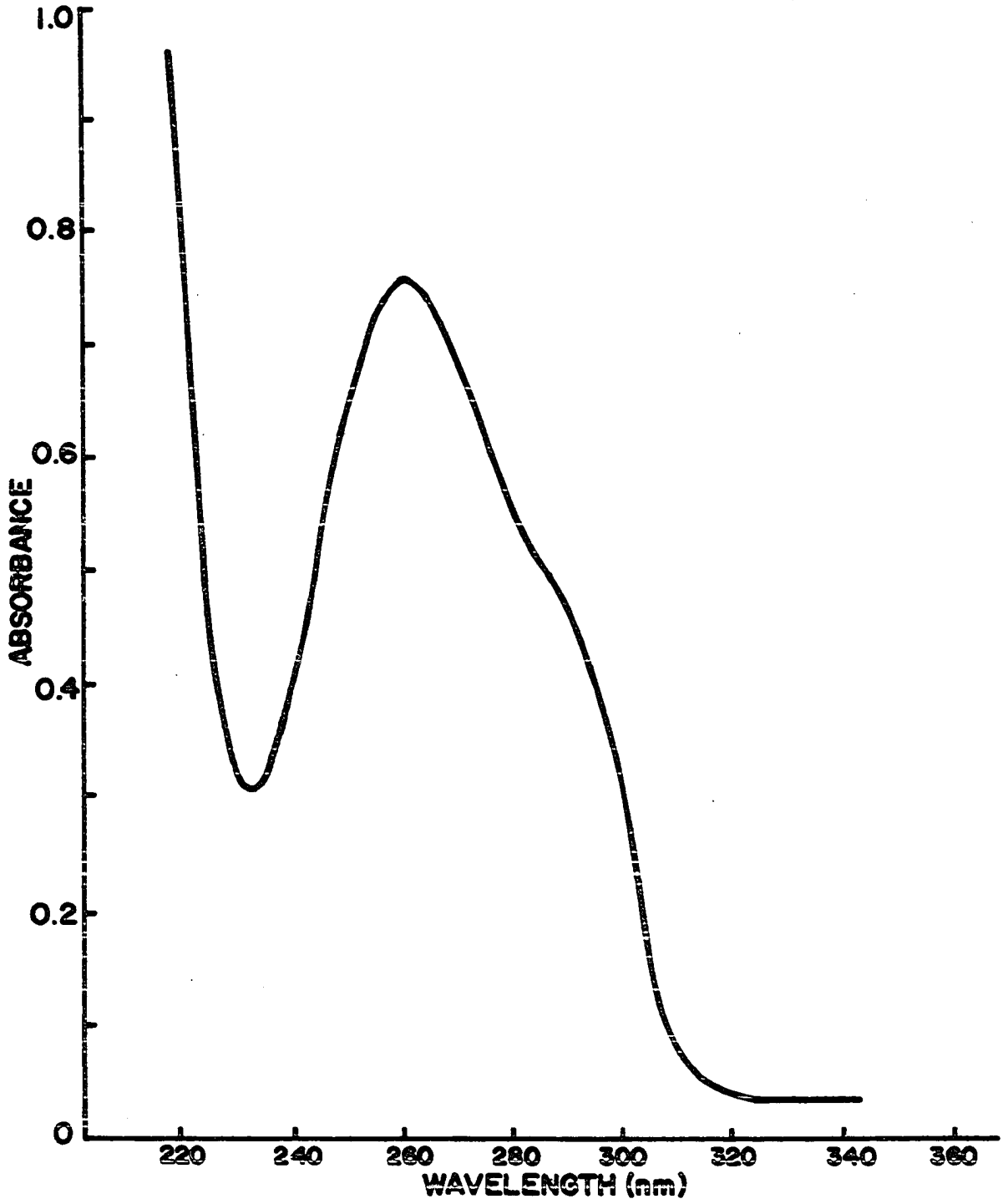

Figure A-10. HMBOA-glucoside

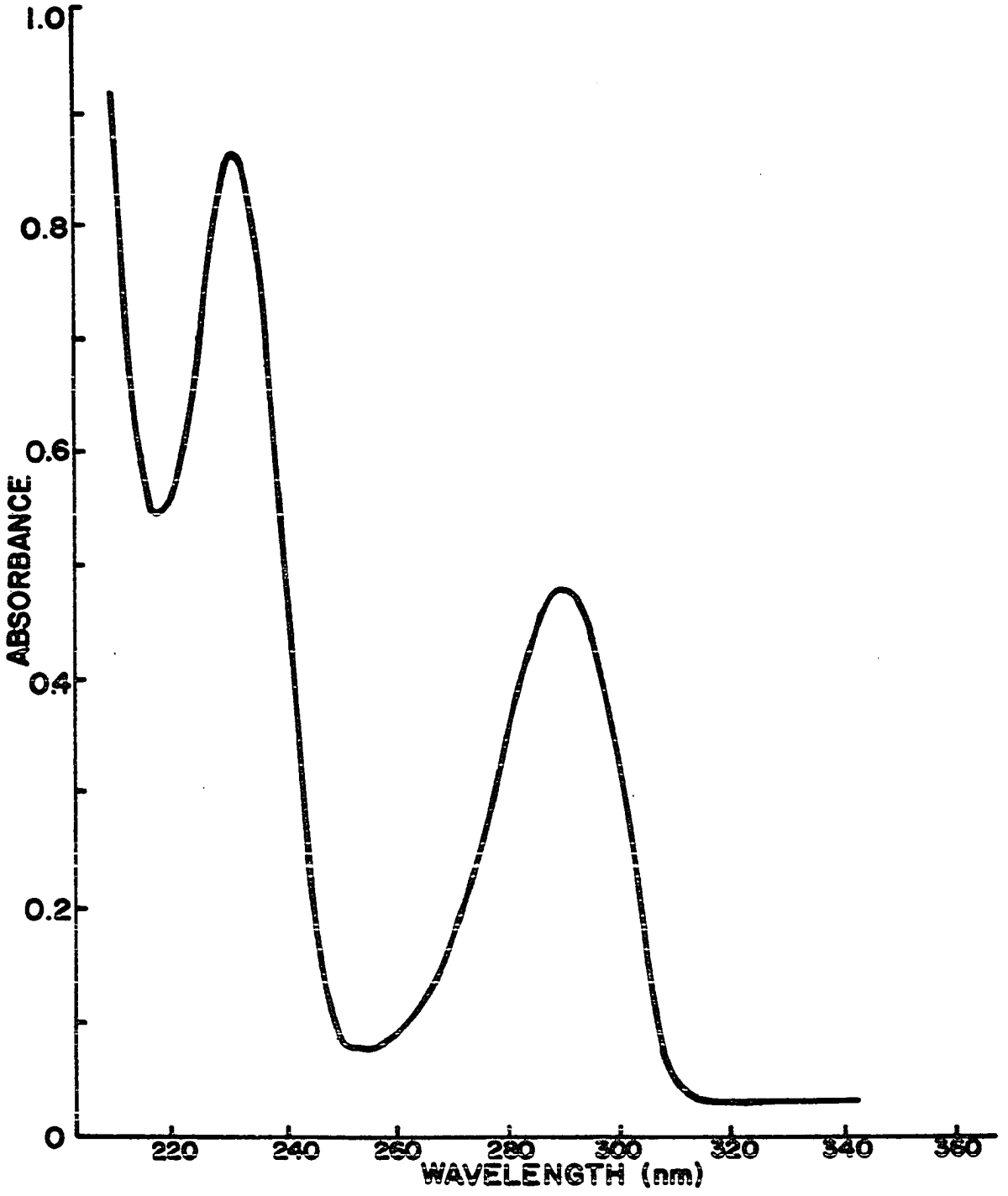

Figure A-11. 6MBOA

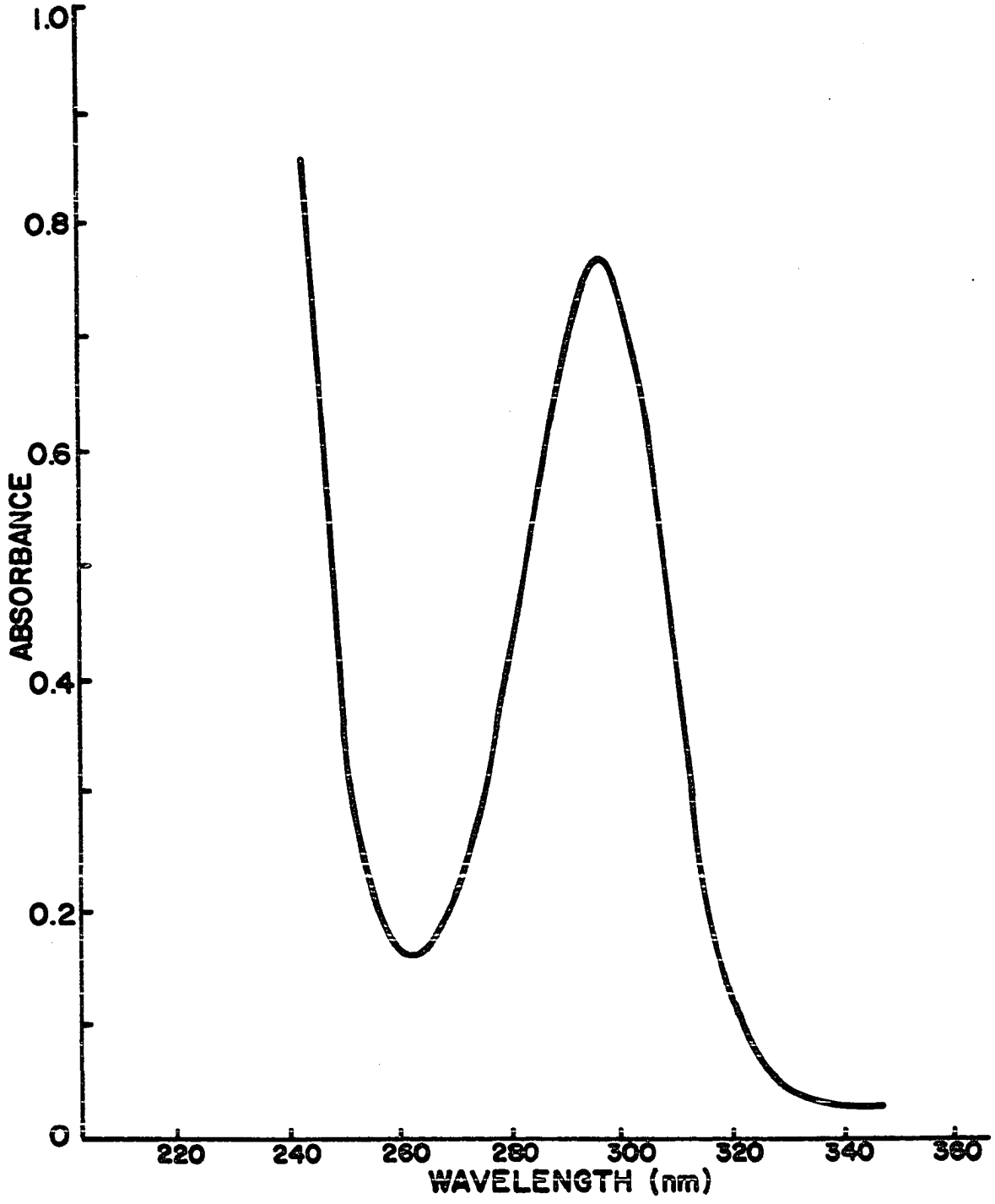

Figure A-12. 5-Methoxy-o-aminophenol

Table A-1. Extinction coefficient (ϵ) of the compounds of Figures A-1 - A-12.

	M.W.	in 95% Ethanol nm	ϵ	in IN HCl nm	ϵ
o-Aminophenol	109	232 286	5519 2568	270	2055
Anthranilic acid	137	248 337	6712 4452		
BOA	135	275	4615		
DIBOA	181	253 282	8150 5022		
DIMBOA	211	263	10490		
Ethyl-o-nitrophenoxy-fluoroacetate	243	252	4913		
GAP	167	243 285	21410 9893		
HBOA	165	250	8954		
HMBOA	195	258	10440		
HMBOA-glucoside	258	260	10700		
6MBOA	165	232 290	10143 5550		
5-Methoxy-o-aminopheno	139	296	3627	275	2586